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Physiological Zoölogy

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STUDIES ON AMPHIBIAN NEOTENY. I. THE METAMORPHOSIS OF THE COLORADO AXOLOTL BY INJECTION OF INORGANIC IODINE

(One plate)

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THE fact that the metamorphosis of anuran larvae may be accelerated by administration of inorganic iodine was demonstrated by the experiments of Swingle (1918–19). Such an acceleration is much more readily produced by the use of organic iodine compounds such as 3, 5 diiodotyrosine, iodoserumalbumin, iodoserumglobulin, iodized casein, iodogliadin, iodoedestin, and iodofibrin, very large quantities of elemental iodine, comparatively, being required to cause the same effects. The metamorphic activity of inorganic and organic iodine compounds other than thyroid iodine, and such as those above mentioned, is at present obscure, but appears to be due to the linkage of iodine to tyrosine or tyrosine derivatives. This seems probable in view of the fact that thyroxin itself is a tetraiodo derivative of tyrosine. Attempts to seek further information regarding the activity of iodine in accelerating metamorphosis have led to experiments with urodele larvae, since metamorphosis in these forms is less complex than in anurans. Jensen (1921) was able to bring about the metamorphosis of the Mexican axolotl by administration of iodized casein, iodoserumglobulin, and iodoserumalbumin, but was unsuccessful when inorganic iodine was used. Hirschler (1922), having metamorphosed

anuran tadpoles by introducing small crystals of iodine into the body, succeeded in metamorphosing one axolotl by injecting iodoform paste, and one by injecting a solution of iodine in potassium iodide. In no other cases was he successful, and in consequence his work has been severely criticized. Huxley and Hogben (1922) and Huxley (1925) obtained no positive results upon feeding iodine to axolotls, although Huxley describes what Spaul calls "premetamorphic symptoms." Uhlenhuth, in view of his experiments with salamanders (1921–22), believes he has conclusively shown that metamorphosis of urodeles is not accelerated by administration of elemental iodine. However, Blacher and Belkin (1927) implanted iodine crystals in the body cavity of the Mexican axolotl with subsequent metamorphosis. These workers likewise succeeded in initiating metamorphosis in hypophysectomized axolotls. These latter experiments, carried out on a neotenic form, are striking, though they have been dismissed by Spaul (1928) as merely possible with "susceptible strains" of axolotls.

In view of the previous discussion, it was thought desirable to conduct similar experiments with the Colorado axolotl, a form which spontaneously metamorphoses when removed from its natural habitat but which does not metamorphose when either thyroidectomized or hypophysectomized.

The writer wishes to express his indebtedness to Dr. W. W. Swingle, who suggested the problem, for his aid and suggestions.

MATERIAL AND METHODS

The axolotls used were of the Colorado type, 6–8 inches long, and when received showed no signs of metamorphosis.

Since this strain of axolotl metamorphoses spontaneously when removed from its native habitat, it was necessary to use hypophysectomized and thyroidectomized individuals for the experimental work. The operation for removal of the pituitary is quite simple. Under strong illumination the anterior lobe is visible through the roof of the mouth, just posterior to the optic chiasma and between the large lobes of the lime-filled endolymphatic sacs. With the point of a strong, sharp-pointed knife a small piece of the cartilaginous floor of the cranium directly beneath the hypophysis is pried

back. Then, if slight pressure is applied to the surrounding area, the gland usually protrudes through the opening and is easily sliced off. Many times the posterior and intermediary portions are removed along with the anterior, in which cases the animals assume a beautiful silvery-white color. In older animals, with harder cranial floors, greater difficulty is encountered, and part, or all, of the posterior and intermediary portions may be left behind. The removal of the anterior lobe, however, is the essential thing for preventing normal metamorphosis. The excised gland was in every case examined with the binocular microscope to make sure of the completeness of the operation. When kept in clean, running water the animals recovered very quickly.

For removal of the thyroids, a binocular microscope is necessary. A median incision is made from the hyobranchial region to the tip of the mandible. The flaps of skin are drawn aside and the thyroids easily located upon the large blood vessels in the triangle formed by the kerato-hyoid and genio-hyoid muscles. The glands may then be dissected away from the blood vessels with a sharp spear-head needle and removed with forceps. Usually a slight hemorrhage results. The wound is closed by suturing the flaps of skin along the median line. The animals must be carefully tended following the operation, as infections, with subsequent fatality, are frequent.

Finely powdered iodine crystals were injected beneath the skin between and anterior to the gills. Sufficient iodine was usually introduced to leave a brown spot from $\frac{1}{4}$ to $\frac{1}{2}$ an inch in diameter. As would be expected, such treatment frequently resulted in death, but it was thought that this area would be better suited to the injection than is the body cavity (Blacher and Belkin reported quite a high mortality due to their intra-abdominal implantations). The animals were not all treated at one time, on account of the difficulty in securing and keeping axolotls. From time to time certain individuals were operated, kept for a period, and then injected. For controls, series of normal and of hypophysectomized animals were kept. Since thyroidectomized axolotls have not been known to metamorphose (Swingle, 1923) these animals were used only for injection, after sufficient time had been allowed for the wounds to heal.

RESULTS

The first group of axolotls was hypophysectomized March 21. Of these, two were injected with iodine March 30, and one was accidentally killed April 24. The three remaining, except one which died May 1, were kept until May 23, when they were injected with iodine. None of these animals showed any signs of metamorphosis, and all were "albinos." April 26, five 8-inch axolotls were hypophysectomized. One of these was taken for injection May 9. The others were kept until June 20, when one of them was killed for examination of the hypophyseal region, two were injected with iodine, and one was kept. None of these metamorphosed. One of this last group showed a slight atrophy of the anterior part of the tail fin before hypophysectomy, but subsequent to the operation showed no further metamorphic symptoms. None of the hypophysectomized animals metamorphosed.

The normal animals began to metamorphose May 1. The changes followed quite rapidly, and by June 7 all had reached adult condition.

The first two hypophysectomized individuals were injected March 30. One died the following day, but the other survived and by April 10 exhibited almost complete atrophy of the tail fin and extensive resorption of the gills. By April 23 it had almost lost its gills, its skin was rough, its eyes protruded, and its tail fin had completely disappeared. A few days later it had left the water. This animal was kept until June 23. It assumed the pigment pattern of a normal adult, although the black areas did not attain the depth of color shown by the normal individuals. From this it was judged that a fragment of pars intermedia had been left and had hypertrophied. The animal was killed and the base of the brain examined and sectioned. A very minute nodule of tissue was found, which upon sectioning proved to be part of the pars intermedia and pars tuberalis. These parts have never been shown to be concerned in amphibian metamorphosis (Swingle, 1921; Atwell, 1921).

The next group of hypophysectomized animals treated failed to survive. May 9, another pituitaryless individual was injected and by May 21 was metamorphosing. It was practically completely metamorphosed when it died, June 7. This animal was recovered too late for autopsy.

May 23 two of the axolotls operated March 21 were injected. One died the next day, but the other transformed quite rapidly and by June 14 was almost completely metamorphosed. It died shortly after, before the final color changes had taken place. This had been an albino, and although the final molt had not occurred, it was still fairly light in color. Autopsy showed a fragment of tuberalis tissue remaining.

Two axolotls which had been hypophysectomized April 25 were injected with iodine June 20. These were not clear albinos, and examination of one of the same group showed a small bit of what was, apparently, pars intermedia tissue. One of these, before operation, showed a slight atrophy of the anterior portion of the tail fin, but following the operation no further symptoms developed until after treatment. June 27 these two animals were beginning to metamorphose, and by June 30 exhibited marked shrinking of gills and tail fin. The especial one just mentioned was farthest advanced at this time. July 4 both injected animals had left the water, and except for pigment changes were completely metamorphosed. An untreated hypophysectomized axolotl of the same group showed no metamorphic symptoms whatever. It was not thought necessary to kill and autopsy these animals, since they had been kept, long after the metamorphosis of the normals, with a hypophysectomized individual of the same group for a further control.

The first thyroidectomized axolotl was injected April 11. Several others treated a few days later failed to survive more than a week. April 23 this animal displayed marked metamorphic symptoms, and when it died, May 1, was practically completely transformed, with the adult pigment pattern beginning to develop. Autopsy showed no thyroid tissue remaining, although the throat region was very carefully examined.

The other individual used was thyroidectomized May 15 and kept until the normals were all advanced in metamorphosis. It was injected with iodine May 25. By June 10 it had left the water and had nearly lost all traces of gills. It was killed for examination June 23, although the final molt had not taken place and the gill region still protruded. Upon autopsy, no thyroid tissue could be found.

Three normal animals were also injected with iodine. Two died

shortly after the injection, having become infected with fungus. The one remaining displayed an accelerated beginning metamorphosis which seemed to slow down subsequently, and the animal transformed no more quickly than did the normals.

DISCUSSION

An explanation for the action of inorganic iodine in inducing amphibian metamorphosis must still be hypothetical. It seems evident from the work done heretofore that iodine is necessary for the normal development of these animals, but it has been claimed by various investigators that the iodine has its effects only when in certain organic combinations. Most of the work has been done upon individuals with thyroids intact. Thyroidectomized animals, while readily transformed by administration of organic iodine, only metamorphose under the influence of inorganic iodine when very large quantities are used. The relationships between the thyroid and iodine are well known. Animals with intact thyroids undoubtedly incorporate any iodine administered into the thyroid hormone, which, when released, causes metamorphosis. In the case of thyroidless animals, the theory has been advanced by Swingle that the iodine to be effective must enter into organic combination with the proteins of the body or with certain amino-acids derived from these proteins. It is not at all unreasonable to assume that such must be the case, since such amino-acids as tyrosine and such proteins as serumalbumin and serumglobulin are readily available in the body; and such substances, when iodized, have been very effective in inducing metamorphosis in both pituitaryless and thyroidless anurans and in thyroidless urodeles (Allen, 1919; Swingle, 1919–23).

Neotenic forms such as the axolotl are known to possess fully developed thyroids; and according to Swingle, it is probable that such animals are not transformed when fed inorganic iodine because the thyroid picks up the iodine but does not release the resulting hormone. The release of this hormone now appears dependent upon the activity of the anterior lobe of the pituitary. However, here we have pituitaryless axolotls metamorphosing under the influence of large quantities of inorganic iodine, and it is well known that Allen (1919) succeeded in transforming pituitaryless tadpoles by adminis-

tration of starch-iodide. If the iodine is taken up by the thyroid, how then is the hormone released? Perhaps the large quantity of iodine introduced so stimulates the thyroid that it forms an excess amount of the hormone which cannot be held in check; or else the surplus iodine forms other effective organic iodine compounds with the other tissues of the body.

The condition of the thyroids of the treated pituitaryless animals is not to be discussed here, though it may be said that macroscopically they appeared larger and more prominent than in the normal larvae.

It would seem clear that the views of Uhlenhuth, Jensen, and Huxley and Hogben, that inorganic iodine will not bring about metamorphosis of tailed amphibians, do not hold in light of experiments conducted as were those of Blacher and Belkin and of those just described. As Blacher and Belkin point out, discrepancies in results may be due to nothing more than differences in the amounts of iodine administered. To this may be added the differences in availability of the iodine administered. It seems evident that feeding inorganic iodine to axolotls does not produce metamorphosis but that intraperitoneal and subcutaneous injections of elemental iodine do markedly accelerate the differentiation processes of these neotenic forms.

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PLATE I

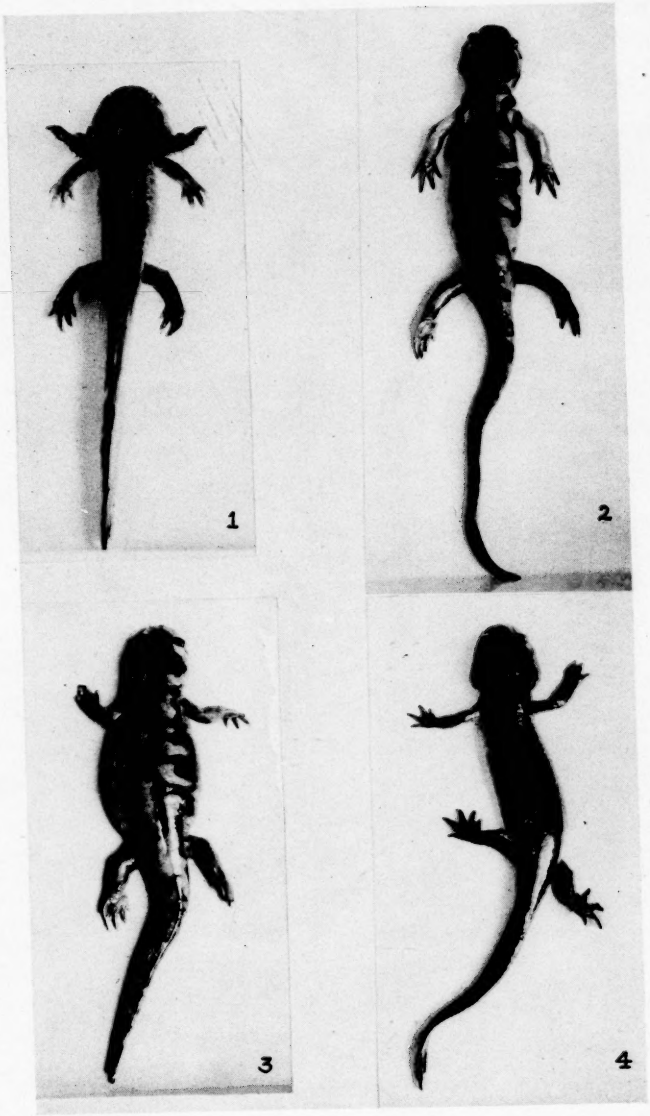
FIG. 1.—Colorado axolotl before injection with iodine.

FIG. 2.—Colorado axolotl, normal metamorphosis.

FIG. 3.—Hypophysectomized axolotl, metamorphosed following injection with crystals of inorganic iodine.

FIG. 4.—Thyroidectomized axolotl, metamorphosed following injection with crystals of inorganic iodine.

PLATE I



A COMPARISON OF SPONTANEOUS ACTIVITY OF THE ALBINO RAT WITH THE ABILITY TO LEARN, WITH SPECIAL REFERENCE TO THE EFFECT OF CASTRATION AND OVARIECTOMY ON THESE PROCESSES[1]

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THE present investigation is directed toward the establishment of the relationship between spontaneous activity and the ability to learn, with special reference to the effect of castration and ovariectomy on these processes.

The importance of the endocrine organs in physiological behavior is so well established that it need not be discussed in detail here. However, just what part these glands play in the learning process is not quite clear.

The spontaneous activity of the albino rat and factors affecting it have been studied from almost every angle by a number of investigators. Since the writers have in mind including in a program of research endocrine organs other than the sex glands, attention is directed to the investigations bearing directly on this subject.

A limited amount of experimentation has been done involving the hypophysis and its relation to spontaneous activity. Hoskins (1927) reports that the injection of an extract of this gland into rats normally showing a low activity caused no change in this behavior. Richter (1927) obtained no change in activity when the hypophysis was removed.

The relation of the thyroid glands to spontaneous activity has been studied in somewhat more detail. Liddell (1923), in attempting to study this problem in sheep, obtained no satisfactory results. Hoskins (1927), Richter (1927), and Lee and Van Buskirk (1928) report that the extirpation of the thyroid glands has no effect on spontaneous activity.

The relation of the adrenal glands to spontaneous activity has

[1] The expenses of this investigation were defrayed, in part, by a grant from the Committee on Research in Problems of Sex, of the National Research Council.

been studied by Durrant (1924) and Richter (1927). Both of these investigators obtained a marked decrease in activity when the glands were removed.

The effect of the sex glands on spontaneous activity has been studied most extensively. That castration and ovariectomy lessen this type of response in adult rats have been shown by Hoskins (1925), Richter (1927), and others.

The effect of the implantation of the sex glands has been reported by a number of investigators. The work of Hoskins (1925) gave negative results when testes were transplanted into the castrate. On the other hand, Richter and Wislocki (1927) reported that testicular grafts cause an immediate return to normal when transplanted into castrated males. Wang, Richter, and Guttmacher (1925) have shown that the transplantation of ovaries into the castrated male caused an even greater increase in activity than the testicular substance.

The investigations, up to the present time, seem to have shown conclusively that castration and ovariectomy cause a marked decrease in spontaneous activity of the adult rat. Furthermore, it is evident that the transplantation of testicular and ovarian substance causes a marked increase in the activity of the castrated animal. The experimental evidence involving endocrine organs other than the sex glands is either too meager or so contradictory that the results are not at all conclusive.

The rôle of the endocrine organs in the learning process is not yet well established or understood, nor has it been thoroughly investigated. However, an experiment dealing with the effect of thyroidectomy on the albino rat, together with the feeding of thyroid gland substance and the injection of thyroid extracts, upon the learning process has been reported by Rickey (1925). This experiment showed, in general, that the extirpation of the thyroid glands caused a marked reduction in the ability to learn. Furthermore, the experiment showed that thyroidectomy caused a progressive incoordination of behavior, which was evident from the fact that movements became slower, less stable, and meaningless. There was also a loss of whatever pre-operative skill or learning that had been ac-

quired. Vitality was reduced, sensibility decreased, and there was a progressive deterioration of the vegetative processes.

The effect of feeding thyroid gland substance and the injection of thyroid extract as reported by Rickey was an appreciable decrease in learning ability accompanied by rapid disco-ordinated movements, loss of skill and accuracy, and inability to acquire new responses.

The experiment of Rickey suggested to the writers, since very definite physical disturbances were reported, that it might be possible that there was a direct relationship between spontaneous activity and ability to learn. If such a relationship exists, then one would expect to find a high correlation between spontaneous activity and learning, not only in normal animals but also in those whose behavior had been modified by the extirpation of endocrine glands or by the feeding of these substances. In view of this fact, a series of experiments are being carried out in which methods that have proven to be effective are being used to modify the activity of animals.

In this experiment castration and ovariectomy are used as a means of modifying spontaneous activity. This method was adopted in our first investigation, not only because it has been proven effective beyond question but also because the operation is simple and does not seriously impair the general health of the animal.

The investigation of Gans (1927) shows that when castration is done before the age of puberty (twelve days) activity is unaffected. Wang (1923) and Sloanaker (1924) report that when rats are spayed before the age of puberty, activity is not only unaffected but remains lower than normal after puberty is reached. These investigations suggested to the writers that it might be well to carry out two experiments, one before puberty and one after. This method of approach was adopted. The present paper deals with that part of the experiment which was carried out before puberty.

Five litters of albino rats, fifteen days of age, were selected for the experiment. Litter 1 contained four males and four females. Two of each sex were operated, but one operated female died before the experiment was completed. Litter 2 contained eight males, four of which were castrated. Litter 3 contained six males and two fe-

males. Three of the former and one of the latter were operated. However, one of the castrated males was killed before the experiment was finished. Litter 4 contained five males, three of which were castrated. Litter 5 contained three males and five females. Two of the former and three of the latter were operated.

The usual method of castration was employed. Both testicles were carefully removed and examined so as to make sure that none of the gland was left. Surgical cleanliness was employed in the operation. One stitch was taken in the scrotum to close the wound.

Ovariectomy was performed as described by Haterius (1928). An incision was made through the back of the animal approximately over the ovary. The gland was carefully removed and examined under the microscope so as to make sure that it had all been removed. Sufficient stitches were taken to close the wound. Ether anesthesia was used in all operations.

Activity was measured by the revolving-cage method as described by Durrant (1924). This apparatus consists of a revolving cage which is attached to a pedometer so that each revolution is recorded. The rat has free access to a feeding-compartment so that he may enter at any time.

The ability of the rats to learn was determined by use of the maze described by Rickey. It is of simple type, providing a pathway from the entrance to the center providing six culs-de-sac, and thus seven possibilities of error. A large glass mirror placed over the maze at an angle of 45° reflects it upon a smaller mirror which is visible to the experimenter seated at the recording-table. The lens and ground glass are eliminated since they were found unnecessary. The maze was artificially lighted, and all experiments were carried out in a dark room. Food was used as a stimulus for the maze-running. The regular ration was placed in the center of the maze, and food was kept from the animals except when they entered this compartment.

Our criterion for learning was more severe than that described by Rickey. She assumed that whenever the animal ran from the entrance to the center in 60 seconds, the maze was learned. In this experiment each animal was required to run the maze three consecutive times without error before the learning was considered as com-

plete. It was found that when this was done, less than 10 seconds were required. This criterion for learning was adopted since a preliminary experiment showed that this was well within the limits of the animals used.

As an index to spontaneous activity the average daily number of revolutions voluntarily run by an animal over a period of 10 days is used.

Ten days after the operation was done the learning ability of each animal was determined. The general method of procedure was as follows: Each litter was given its ration of food in the center of the maze on the night preceding the beginning of the experiment. On the night following, each animal was placed in a starting-box provided with a sliding door which was controlled by the experimenter from the recording-table. When all was in readiness, the door was opened and the animal was allowed to find his way through the maze to the feeding compartment. When this was reached, he was allowed to eat in a normal manner. Time was determined in seconds by a stop watch. The experiment was continued on consecutive nights until the criterion for learning was met.

As soon as the learning experiment was completed, each litter was placed in the activity cages. A 24-hour period was allowed for practice and also for the purpose of allowing the animals to become accustomed to the cages. They were provided with as much of the regular ration as was desired.

Data were collected from five litters of rats containing thirty-five animals. Fifteen were operated, and twenty were used as controls.

The data are presented in tables according to litters. In the experiment dealing with spontaneous activity, the figures in the column marked "Activity" refer to the average daily number of revolutions run over a period of 10 days. In the learning experiment, data were collected regarding the number of trials required to learn the maze, the number of errors made in so doing, and the total time required for the learning process.

Table I shows the data collected from litter 1 and reveals the fact that on the average the castrates required one more trial than the controls, but made five less errors. The castrates learned the maze in 6 less seconds but ran 185 less revolutions. These data indi-

cate, as far as learning is concerned, that castration had no effect. The difference in activity is so slight that it is of no significance.

TABLE I

DATA COLLECTED FROM LITTER 1

RAT	NO. OF TRIALS		NO. OF ERRORS		TIME IN SECONDS		ACTIVITY	
	Controls	Castrates	Controls	Castrates	Controls	Castrates	Controls	Castrates
1♂	.....	25	.....	33	.....	665	.....	1,412
2♂	.....	11	.....	20	.....	290	.....	2,625
3♀	.....	26	.....	51	.....	481	.....	2,538
4♂	15	.....	32	.....	437	.....	1,200	.....
5♂	17	.....	42	.....	367	.....	4,200	.....
6♀	24	.....	49	.....	608	.....	2,427	.....
7♀	22	.....	38	.....	524	.....	1,678	.....
Average....	19	20	40	35	484	478	2,376	2,191

Table II shows the data obtained from litter 2 and reveals the fact that the number of trials required to learn the maze was the same for both controls and castrates. However, the castrates made

TABLE II

DATA COLLECTED FROM LITTER 2

RAT	NO. OF TRIALS		NO. OF ERRORS		TIME IN SECONDS		ACTIVITY	
	Controls	Castrates	Controls	Castrates	Controls	Castrates	Controls	Castrates
1♂	16	.....	51	.....	428	.....	1,586	.....
2♂	17	.....	81	.....	1,216	.....	Died	.....
3♂	.....	15	.....	44	.....	412	.....	2,624
4♂	.....	11	.....	39	.....	528	.....	2,952
5♂	.....	18	.....	67	.....	1,332	.....	1,462
6♂	.....	18	.....	62	.....	720	.....	3,317
7♂	18	.....	80	.....	360	.....	3,486	.....
8♂	11	.....	57	.....	529	.....	Killed	.....
Average....	15	15	67	53	633	748	2,536	2,588

14 less errors than the normal animals. The time required to learn the maze was in favor of the controls by 115 seconds, while the castrates were more active by 52 revolutions. This litter, as in the case of litter 1, appeared to be unaffected by castration with regard to both activity and learning.

Table III shows the accomplishments of litter 3. The data col-

lected from litter 3 indicate that the castrated animals required 4 more trials to learn the maze and made 19 more errors. They learned more slowly, on the average by 196 seconds, and were less active

TABLE III

DATA COLLECTED FROM LITTER 3

RAT	NO. OF TRIALS		NO. OF ERRORS		TIME IN SECONDS		ACTIVITY	
	Controls	Castrates	Controls	Castrates	Controls	Castrates	Controls	Castrates
1♂	26	.....	67	.....	472	.....	2,881	.....
2♂	3	.....	18	.....	376	.....	2,042	.....
3♂	17	.....	52	.....	360	.....	2,296	.....
4♂	.....	26	.....	90	.....	608	.....	1,927
5♂	.....	11	.....	48	.....	468	.....	2,421
6♀	.....	19	.....	47	.....	592	.....	1,510
7♀	11	.....	33	.....	234	.....	4,546	.....
Average	14	18	42	61	360	556	2,941	1,952

by 989 revolutions. For this litter the normal animals had a decided advantage in every phase of the experiment.

Table IV contains the data collected from litter 4, where we find that the normal animals required 14 more trials, on the average, to

TABLE IV

DATA COLLECTED FROM LITTER 4

RAT	NO. OF TRIALS		NO. OF ERRORS		TIME IN SECONDS		ACTIVITY	
	Controls	Castrates	Controls	Castrates	Controls	Castrates	Controls	Castrates
1♂	24	.....	54	.....	476	.....	3,261	.....
2♂	26	.....	50	.....	396	.....	1,570	.....
3♂	.....	21	.....	29	.....	228	.....	2,720
4♂	.....	5	.....	33	.....	432	.....	2,708
5♂	.....	8	.....	28	.....	159	.....	3,378
Average	25	11	52	30	436	273	2,415	2,935

learn the maze. They made 22 more errors during the learning process. The normal animals required 163 seconds more time and ran 520 less revolutions. Here the conditions were just the reverse of those in litter 3, for the castrates had a decided advantage.

Table V contains the data collected from litter 5. The data show that the control animals required 4 less trials and made 16 less errors in learning the maze. They required 126 seconds less to complete

the learning and ran 807 more revolutions. Here we find that the normal animals learn faster, require less trials, make fewer errors, and are more active than the castrates.

TABLE V

DATA COLLECTED FROM LITTER 5

RAT	NO. OF TRIALS		NO. OF ERRORS		TIME IN SECONDS		ACTIVITY	
	Controls	Castrates	Controls	Castrates	Controls	Castrates	Controls	Castrates
1 ♀	.....	23	.....	65	.....	510	.....	1,134
2 ♀	.....	12	.....	28	.....	499	.....	1,002
3 ♂	.....	14	.....	28	.....	369	.....	1,172
4 ♂	.....	11	.....	55	.....	474	.....	1,330
5 ♀	8	.....	24	.....	470	.....	1,223	.....
6 ♀	7	.....	30	.....	247	.....	3,473	.....
7 ♂	20	.....	35	.....	324	.....	1,420	.....
8 ♀	.....	15	.....	51	.....	515	.....	1,511
Average....	11	15	29	45	347	473	2,039	1,232

When considered from the standpoint of group averages, the data show quite clearly that there is a relationship between the ability to

TABLE VI

THE RELATION BETWEEN ABILITY TO LEARN AND SPONTANEOUS ACTIVITY

LITTER 1		LITTER 2		LITTER 3		LITTER 4		LITTER 5	
Learning	Activity	Learning	Activity	Learning	Activity	Learning	Activity	Learning	Activity
1	2	1	1	1	1	1	1	1	1
2	1	2	4	2	3	2	4	2	2
3	7	3	3	3	5	3	5	3	5
4	3	4	5	4	4	4	3	4	4
5	5	5	2	5	2	5	2	5	3
6	4	6	6	.....	.....	6	7	6	7
7	6	.....	.....	.....	.....	7	6	7	6

learn and spontaneous activity. Furthermore, it is evident that castration before puberty affects neither learning nor spontaneous activity.

In order to get a clearer conception of the relationship between the processes under consideration, it is evident that an individual study should be made. In Table VI the data are presented in such a manner that the relationship between the ability to learn and spontaneous activity is shown. In each litter the individual rats are ranked according to the speed of learning the maze. Opposite the

learning rank is the rank in activity. For example, the rat in litter 1 which ranked first in learning ranked second in activity.

The arrangement of the data in Table VI makes it convenient to determine the coefficient of correlation between learning and spontaneous activity. This was done by means of Spearman's rho formula.[1] For litter 1 the coefficient of correlation is 0.570; for litter 2, 0.600; for litter 3, 0.300; for litter 4, 0.643, and for litter 5, 0.822. The coefficient of correlation indicates that there is a relationship between spontaneous activity and learning. In case of litter 3 the relationship is slight, while in litter 5 it is extremely high.

Although the correlations are somewhat low in some litters, there is a point of interest brought out by Table VI. The data show that in every case except one the animals which were most active were the most adept at learning the maze. The exception was in litter 2, and here the animal most adept at learning was second in spontaneous activity. Furthermore, the data show that the animals requiring the longest time to learn the maze were the less active except in litter 3. Here the animal ranking second in learning was fifth in activity. The data show that there is but little relation between activity and learning in the intermediate group. This is accounted for by the fact that the differences were too slight to bring out any outstanding variations.

The fact that castration before puberty has no effect on spontaneous activity is in accordance with other investigations of this nature. The lack of any evidence that castration of young animals affects their learning before puberty is in accordance with the findings relative to spontaneous activity.

It has occurred to the writers that it might be possible that our criterion for learning was too severe. However, if 60 seconds were used as learning time, as was the case in the experiment of Rickey, our animals showed evidence of lack of definite knowledge of the correct path from the entrance box to the feeding-compartment; in fact, in many cases this time was attained after the second or third trial. In order to find a time criterion well within the limits of our rats, a preliminary experiment was carried out which substantiated our idea as to the criterion used by Rickey.

[1] $\rho = 1 - \frac{6\Sigma D^2}{N(N^2-1)}$. D = Difference in rank on the two tests. N = Number of cases.

There is some question in the minds of the writers relative to the effects of the extirpation of glands of internal secretion on the learning process. Although there were no physical disturbances developed in the animals used in this experiment, it seems reasonable to suppose that since these glands play such an important rôle in physiological function, alteration in psychological behavior, such as learning, is not the result of a direct mental disturbance due to the extirpation of a gland but is more likely an indirect effect due to physiological disturbances. This idea might be investigated by comparing learning with spontaneous activity. Although the writers do not overlook the fact that the extirpation of the thyroid gland has not been shown to alter spontaneous activity, it would seem that animals suffering from such severe physical symptoms as reported by Rickey would show an alteration of their spontaneous activity.

Since the speed of conduction through the reflex arc has been shown by Travis (1928) to be an index of intelligence, it does not seem unreasonable to carry such technique over into animal behavior. It would be of interest to know whether the ability of an animal to learn depends on the same factors as those involved in human intelligence. By employing a similar technique, it is entirely possible to approach the learning problem in the same way as has been done in the case of intelligence.

It is the intention of the writers to investigate the relationship of spontaneous activity to the ability to learn, not only as affected by castration but also as affected by other endocrine glands.

CONCLUSIONS

Five litters containing thirty-five rats under fifty days of age were used as the basis for comparing the ability to learn a simple maze with spontaneous activity. In order to alter the spontaneous activity, fifteen animals were castrated. Twenty were used as controls. The data show the following:

1. Castration does not alter the spontaneous activity of the albino rat before puberty is reached.

2. Castration does not alter the ability of the albino rat to learn a simple maze before puberty.

3. The correlation between spontaneous activity and learning shows that there is a relationship between these two processes.

4. The data show that the most active animals learn most rapidly and the least active animals learn least rapidly.

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ANTIBODY FORMATION AGAINST AN ANIMAL'S OWN TISSUE

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INTRODUCTION

IT IS common bacteriological practice to produce antibodies in an animal against injected foreign proteins, but it is comparatively recent work that shows that an animal can produce antibodies against its own tissues. Dr. M. F. Guyer, under whose direction this work was conducted, showed in 1925 (*Journal of Infectious Diseases*, V, 37) that an animal could build antibodies against its own displaced lens tissue. By "needling" the crystalline lens of the rabbit, sufficient lens material was set free in the blood stream to immunize the animal. The precipitin test gave a definite cloudiness when a solution of pulverized lens was used as an antigen; but as long as the lens tissue was undisturbed, all tests were negative. Although it was being nourished all the while by the blood, it was not until the lens was injured or destroyed that it entered the blood stream and immunized the animal.

The purpose of this investigation was to determine, if possible, whether or not less specialized tissue, or a tissue more affected by changing environment, such as skin, could be made to produce antibodies in the animal which bore it. Skin was destroyed on rabbits with ultra-violet light produced by a mercury-vapor quartz lamp. The presence of the skin antibody was shown by the standard bacteriological precipitin test. In later work the same thing was attempted in guinea pigs, and the anaphylactic reaction was used to determine the presence of the antibody. The skin protein used for testing the blood of the immunized animals was made by pulping the dried skins of newly born rabbits and extracting overnight in Ringer's solution (1 gr. of skin to 5 cc. of Ringer's). The protein filtrate thus obtained gave the Heller's protein test in a dilution of 1 to 150 parts.

EXPERIMENTS AND METHODS

To find if the skin antigen was reactive, 5 cc. was injected into fowl on three alternate days. The fowl were tested 2 weeks later, and antibody was found to be present. Similar injections into rabbits gave similar results. With this preliminary out of the way, an experiment was devised by which skin tissue of an animal's own body could be sent into the blood stream.

EXPERIMENT I

Two rabbits were irradiated on the ear with the water-cooled mercury-vapor lamp at a distance of about 1 cm. from the ear. A spot about 2.5 cm. in diameter was irradiated on alternate days as follows: 1.5, 2, 3, and 4 minutes respectively. The results are shown in Table I. Although two other animals gave no reaction after ultra-violet-light exposure, this procedure shows the development of an antibody in three as shown in Table I.

TABLE I

RESULTS AS SHOWN BY PRECIPITIN TEST

Animals	Treatment	Precipitin Titer Before	Precipitin Titer After
1	Ultra-violet light	0	400
2	Ultra-violet light	0	800
3	Injection of skin antigen	0	800

EXPERIMENT II

Longer exposure was given to four animals to find if the titer could be increased by more of the same treatment. These animals were given twelve 5-minute treatments in the same manner as described above. The first five treatments were given in 3-day intervals, while the rest were given in 2. In spite of such drastic procedure, however, the titer remained about the same as for the shorter exposure. This is shown in Table II.

EXPERIMENT III

Intense treatment was given to two animals on a clean-shaven area on their sides, where the skin would perhaps be more sensitive to ultra-violet light. Fifteen treatments were given in all, one being

given each day. The first exposure was for less than 1 minute; but for each day thereafter, there was a decided increase in the length of exposure until the last, which was for 35 minutes. Ten days after the first treatment the precipitin test was run, and the results were much the same as the preceding, the titer being around 800 in both animals.

EXPERIMENT IV

During the course of this work questionable results were obtained in a number of cases. A few animals gave the precipitin reaction previous to treatment; others, however, gave it only following treatment. Such results are to be expected, however, since skin is subjected to so many environmental injuries.

TABLE II

RESULTS AS SHOWN BY PRECIPITIN TEST

Animals	Treatment	Precipitin Titer Before	Precipitin Titer After
A1	Ultra-violet light	0	800
A2	Ultra-violet light	0	400
A3	Ultra-violet light	0	1,600
A4	Ultra-violet light	0	800

Anaphylaxis was resorted to in order to clear up the question, since this reaction is a very definite one. Surely, if an anaphylactic shock could be produced by the injection of skin solution into an animal that had been sensitized to skin by the action of ultra-violet light rays, there would be no doubt as to the production of antibodies.

Guinea pigs were prepared for treatment by clipping the hair closely on their backs, sides, shoulders, and between the ears. For the first treatment five pigs were selected and three were irradiated with the air-cooled mercury-vapor lamp for 1 hour at a distance of 30 cm. from the lamp. The skin became severely burned and after several days began to scale off, and, in extreme cases, small sores appeared. Two weeks from the time they were irradiated, two of the treated animals and one control were injected intraperitoneally, each with 5 cc. of skin solution. One treated animal showed a disturbance; although not violent, it was definitely an anaphylactic shock. There

were respiratory difficulties (sniffling, rubbing the nose, etc.), hiccoughs, trembling, peculiar jumping as though irritated from within, and frequent urination. This lasted several minutes and then the animal became normal again.

Exactly the same set-up was made with eleven other animals, five others being kept as controls. Eight of the treated animals and four controls were injected with the skin solution. Of the eight treated animals, six responded with a mild positive reaction, while the other two and the controls were negative. From these data it would seem that the observations on the precipitins were correct. Two of the treated guinea pigs and one of the controls were bled for precipitin reactions. One treated animal gave a positive test, while the other two did not.

REMARKS

In none of the cases was the anaphylactic reaction violent, as all animals that showed a reaction recovered in a short time and appeared to be quite normal. There were no attempts made to refine this technique and to find the intensity limitations of the anaphylaxis. The only intention of this work was to find if an animal could be sensitized against its own tissue.

CONCLUSIONS

1. These experiments substantiate the idea that animals can build antibodies against their own tissues.

2. Destruction of skin by ultra-violet light causes antibody formation against skin protein.

3. Guinea pigs tend to show anaphylactic reactions to sensitivity brought about by ultra-violet light.

THE EFFECT OF X-RAYS ON THE SPERMATOZOA OF DROSOPHILA

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INTRODUCTION

THE susceptibility of spermatozoa to various physical and chemical agents has been studied by many investigators. Parkes (1925) X-rayed male rats and produced an effect on the sex ratio of the progeny which varied with the interval after X-raying. During the first 5 days a deviation of 2.5 times the probable error was observed, while from the fifth to the eighteenth day a difference of 6.1 times the probable error was obtained. The deviations were in different directions; the first period produced an excess of males; the second, an excess of females.

Muller and Settles (1927) studied the effects of aging on the spermatozoa of *Drosophila* and found no significant difference in the progeny from fresh and old spermatozoa. They also investigated the effect of X-rays on deficient and normal sperm and found a difference which was 3.2 times its own probable error. They concluded that "X-rays probably did cause some difference between the zygote ratios of the treated and non-treated series although this difference was in a direction opposite to that which a selection of sperm on the basis of genetic content should have brought about." For the purpose of their experiment Muller and Settles X-rayed the spermatozoa in the body of the female, and the female germ cells were thus also exposed. In the experiments recorded here, the spermatozoa were X-rayed in the male, and the sex ratio of their progeny was determined by mating treated males to non-treated females. The work was undertaken upon the suggestion of Dr. E. G. Anderson.

TECHNIQUE

Males of an echinus cut[6] garnet[2] stock were treated with X-rays and mated with bar females and also with broad echinus females

having attached *X*'s. All the males used were X-rayed within 24 hours after hatching. The attached-*X* females mated with the X-rayed echinus cut garnet males served as a check on the results of the mating between bar females and X-rayed males. The echinus cut garnet sons resulting from echinus cut garnet ♂ × broad echinus ♀ were exceptional and derived their *X*-chromosome from their fathers and their *Y*-chromosome from their mothers. Similarly, the broad echinus daughters were determined by the *Y*-chromosome of the male. On the other hand, the cross echinus cut garnet ♂ × bar ♀ produced normal males and females. Thus in the one mating the females come from the *Y*-sperm, the males from the *X*-sperm; in the other the males come from the *Y*-sperm and the females from the *X*-sperm. Any differential effect of the X-rays on *X*- and *Y*-sperm should appear in both matings but in opposite sexes. In this way it was possible to some extent to eliminate differences in the sex ratio due to a differential viability of the sexes. The males in *Drosophila* are notably less viable than the females, under ordinary cultural conditions. Warren (1918) gives 95 males to 100 females; Mann (1923), 98.4 males to 100 females.

The effect was examined for a total of 12 days in 3-day intervals, although after the sixth day the numbers are too small to be of significance.

The males were X-rayed for 10 minutes with a Coolidge tube, using a voltage of 130 kilovolts and a current of 5 milliamperes. The distance from the target was 8 inches.

RESULTS

In the first experiment the results of matings between bar females and X-rayed males for the first 3 days gave an excess of males which was a deviation from a 1:1 ratio of 2.8 times the probable error (Table I, A). Statistically, such a difference is not considered very significant, since it might be expected, under conditions of random sampling, once out of 16 trials. However, two things must be taken into consideration. First, the normal sex ratio is not 1:1, since the females are usually more viable than the males. Second, the results, taken bottle by bottle, are very consistent; so that the difference is not due to a large excess of males in one or a few bottles. Out of 40

cultures, 24 showed an excess of males, 2 gave equal numbers of males and females, and 14 produced an excess of females (Table II). This experiment thus indicates a differential deficit of *X*-sperm as against *Y*-sperm.

During the second 3-day period the results were unsatisfactory, and no conclusion could be drawn because of the small number of flies. A deviation of 0.8 times the probable error was obtained, and this was due to an excess of females (Table I, A′).

TABLE I

OFFSPRING FROM X-RAYED MALES

Reference	Female	Days	Cultures	Daughters	Sons	Dev./P.E.
A	B	0–3	40	3,416	3,577	2.8
A′	B	3–6	28	294	280	0.8
B	br ec	0–3	40	1,120	938	6.0
B′	br ec	3–6	28	163	106	5.1
C	B	0–3	87	5,774	6,182	5.5
C′	B	3–6	45	872	979	3.7
D	br ec	0–3	87	2,295	1,826	10.8
D′	br ec	3–6	45	334	279	3.4
A+C	B	0–3	127	9,190	9,759	6.1
A′+C′	B	3–6	73	1,166	1,259	2.8
B+D	br ec	0–3	127	3,415	2,764	12.2
B′+D′	br ec	3–6	73	497	385	5.5
S	B	0–6	200	10,356	11,018	6.7
T	br ec	0–6	200	3,912	3,149	13.4

The first mating of the broad echinus females with attached *X*'s and X-rayed males gave a difference, in the direction of more females, of 6.0 times the probable error for the first 3 days (Table I, B). This deviation from a 1:1 ratio might be expected only about once in 19,230 trials. The deficit of males must have been due to a relative deficit of functional *X*-sperm, since here the male zygotes are determined by the *X*-sperm and the female zygotes contain the *Y*-sperm. This result is the more significant since attached-*X* females usually give fewer daughters than sons (Morgan, Bridges, and Sturtevant, 1925).

The following period, 3–6 days, gave a deviation of 5.1 times the probable error, again an excess of females, which is consistent with all of the previous results (Table I, B′).

The second series of experiments was carried out on a larger

TABLE II

FIRST SERIES

PROGENY FOR THE FIRST 3 DAYS FROM X-RAYED MALES

Bottle No.	Bar ♀ *X*-Sperm	Bar ♂ *Y*-Sperm	Echinus Cut Garnet ♂ *X*-Sperm	Broad Echinus ♀ *Y*-Sperm
101	82	77	19	24
102	174	161	41	37
103	86	86	30	36
104	67	100	17	38
105	73	79	18	16
106	143	137	40	41
107	79	88	42	56
108	144	153	40	40
109	67	91	21	22
110	25	27	6	4
111	76	97	16	15
112	39	33	13	26
113	66	78	27	30
114	58	54	28	26
115	175	177	34	36
116	133	126	15	23
117	42	30	21	38
118	71	92	31	30
119	20	7	10	20
120	145	190	20	35
121	64	60	23	24
122	172	164	28	33
123	82	87	15	22
124	36	45	0	5
125	20	29	28	36
126	44	47	14	18
127	80	95	22	25
128	97	95	22	32
129	91	94	*	*
130	37	37	43	57
131	126	136	25	23
132	91	84	21	18
133	48	47	30	33
134	36	42	14	24
135	92	70	29	25
136	93	101	21	25
137	104	108	24	28
138	146	158	36	44
139	90	92	27	23
140	102	103	27	33
Total (101–40)	3,416	3,577	938	1,120

* Attached *X*'s broke apart.

scale, with the hope of getting more decisive results one way or the other. The data for the first 3 days are recorded in Table I, C. Again there is an excess of bar males, the difference amounting to 5.5 times the probable error. The results, taken bottle by bottle,

TABLE III

FIRST SERIES

PROGENY FOR THE SECOND 3 DAYS FROM X-RAYED MALES

Bottle No.	Bar ♀ *X*-Sperm	Bar ♂ *Y*-Sperm	Echinus Cut Garnet ♂ *X*-Sperm	Broad Echinus ♀ *Y*-Sperm
151	13	10	3	2
152	6	1	24	28
153	45	32	8	17
154	35	28	17	13
155	0	0	9	21
156	3	4	5	6
157	9	8	1	3
158	10	14	1	8
159	3	6	1	6
160	4	1	3	3
161	1	4	0	0
162	3	1	0	1
163	13	8	6	6
164	10	11	2	3
165	1	1	1	2
166	9	11	1	9
167	6	4	5	13
168	12	9	3	4
169	9	15	5	6
170	0	1	2	1
171	1	1	1	1
172	17	16	2	0
173	23	32	1	2
174	4	9	0	1
175	14	7	4	4
176	10	15	3	1
177	7	4	1	2
178	26	27	0	1
Total (151–78)	294	280	106	163

are consistent. Out of 87 cultures used, 55 showed an excess of males, 25 an excess of females, 2 gave equal numbers, and 5 no progeny at all (Table IV).

The second period yielded a difference of 3.7 times the probable error, with an excess of males (Table I, C′).

The attached-*X* females and X-rayed males in the second experiment showed a difference amounting to 10.8 times the probable

TABLE IV

Second Series

Progeny for the First 3 Days from X-rayed Males

Bottle No.	Bar ♀ *X*-Sperm	Bar ♂ *Y*-Sperm	Echinus Cut Garnet ♂ *X*-Sperm	Broad Echinus ♀ *Y*-Sperm
401	52	57	11	10
402	74	73	15	11
403	47	54	16	27
404	77	78	19	31
405	101	105	34	28
406	65	75	24	26
407	83	81	25	29
408	41	49	31	34
409	0	0	47	61
410	54	64	26	29
411	56	58	15	20
412	54	71	46	57
413	31	41	24	37
414	3	5	24	27
415	106	92	19	31
416	31	47	26	22
417	67	60	10	9
418	27	18	22	32
419	49	40	9	10
420	37	44	26	25
421	00	00	11	11
422	1	4	33	31
423	89	112	13	27
424	22	23	11	23
425	35	65	20	23
426	0	0	18	30
427	00	0	32	38
428	44	33	32	46
429	75	80	23	42
430	56	61	2	4
431	33	40	10	16
432	34	41	19	27
433	74	98	27	39
434	134	133	25	34
435	77	81	7	15
436	51	46	19	22
437	112	110	18	15
438	100	85	27	39
439	78	80	25	25
440	80	70	23	25
441	86	93	16	19
442	75	71	12	26
443	87	102	5	4
444	0	00	9	12
445	29	35	27	34
446	94	106	7	12
447	29	33	22	29
448	102	105	17	12
449	52	52	8	22
450	47	64	13	12

TABLE IV—*Continued*

Bottle No.	Bar ♀ *X*-Sperm	Bar ♂ *Y*-Sperm	Echinus Cut Garnet ♂ *X*-Sperm	Broad Echinus ♀ *Y*-Sperm
451	99	95	24	30
452	44	43	36	31
453	110	107	31	44
454	81	94	12	13
455	134	132	21	29
456	82	82	20	42
457	99	80	16	16
458	88	101	7	9
459	110	113	18	21
460	84	85	27	15
461	26	27	22	23
462	58	77	19	40
463	91	94	15	22
464	26	28	9	8
465	122	134	32	47
466	105	101	23	23
467	45	50	19	24
468	77	74	5	20
469	115	122	37	44
470	34	43	14	8
471	84	70	43	50
472	70	68	40	56
473	117	125	25	46
474	142	134	11	19
475	87	98	19	21
476	53	63	43	42
477	71	68	19	19
478	71	101	14	20
479	78	107	31	29
480	42	45	7	16
481	83	107	21	23
482	104	117	35	32
483	104	122	41	55
484	108	122	15	13
485	117	138	20	29
486	60	63	21	32
487	22	17	14	14
Total (401–87)	5,774	6,182	1,826	2,295

error in the first 3 days (Table I, D). Taken bottle for bottle, 62 gave an excess of females, only 19 yielded an excess of males, while 6 showed equal numbers. In this case, moreover, one would normally expect a slight deficit of females (Table IV).

During the following 3 days the deviation was in the same direction and amounted to 3.4 times the probable error (Table I, D′).

Since the results of the two series of experiments are so consistent, the numbers have been totaled for the first 3 days (Table I,

A+C and B+D), for the second 3 days (Table I, A′+C′ and B′+D′), and finally the sum total of the effect has been determined for

TABLE V

SECOND SERIES

PROGENY FOR SECOND 3 DAYS FROM X-RAYED MALES

Bottle No.	Bar ♀ *X*-Sperm	Bar ♂ *Y*-Sperm	Echinus Cut Garnet ♂ *X*-Sperm	Broad Echinus ♀ *Y*-Sperm
501	43	49	9	11
502	8	18	3	3
503	17	14	11	27
504	11	15	2	4
505	1	0	2	2
506	6	8	2	0
507	1	0	6	8
508	69	49	9	3
509	20	20	11	14
510	74	83	5	6
511	38	37	3	3
512	67	50	1	3
513	68	87	0	0
514	35	32	11	12
515	2	2	3	4
516	28	23	2	7
517	7	12	3	3
518	40	61	0	15
519	22	32	0	0
520	10	16	4	5
521	4	8	4	4
522	3	11	1	1
523	1	2	7	9
524	7	10	9	8
525	2	0	2	4
526	22	20	10	10
527	22	24	11	8
528	16	25	1	1
529	0	0	4	1
530	2	6	1	0
531	28	32	6	12
532	21	32	3	8
533	12	6	17	16
534	7	6	9	9
535	25	35	3	4
536	11	14	3	3
537	9	15	20	15
538	12	13	7	4
539	2	0	13	9
540	21	32	9	20
541	14	12	3	4
542	28	25	10	15
543	23	25	11	11
544	13	18	19	19
545	0	0	9	9
Total (501–45)	872	979	279	334

the first 6 days (Table I, S and T). The total progeny of the bar females for 6 days (Table I, S) gives a deviation of 6.7 times the probable error; while for the attached-*X* females (Table I, T), a difference of 13.4 times the probable error is recorded.

SUMMARY AND DISCUSSION

Experiments recorded in this paper make it highly probable that an effect has been produced on the sex ratio in *Drosophila* by treating the males with X-rays. The total deviation from a 1:1 ratio amounted to 6.7 times the probable error in the progeny of bar females and 13.4 times the probable error in the offspring of broad echinus females having attached *X*'s.

No explanation of these results is offered. The data indicate that it is very probable that X-rays produce an excess of normal males and a deficit of exceptional males. This would lead to the inference that there is a differential susceptibility of the *X*-sperm and the *Y*-sperm to X-rays, the *X*-sperm suffering a relative loss in functional activity.

Parkes (1925) found that during the first period after X-raying male rats an excess of males resulted, while during the following period an excess of females was produced. No such tendency toward a reversal of ratios was observed in *Drosophila*. In all the experiments the results were in the same direction, i.e., there was a deficit of individuals resulting from fertilization by *X*-spermatozoa.

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THE NORMAL BLOOD PICTURE OF WHITE RATS AND THE CHANGES IN THE PICTURE FOLLOWING THYROID FEEDING[1]

(One plate and thirteen figures)

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INTRODUCTION

DURING the course of investigations on the thyroid in recent years there has grown up considerable evidence which indicates that hyperthyroidism and hypothyroidism may affect the cellular content of the blood. In amphibian larvae Jordan and Speidel (1923*b*) report an increased haemopoietic action of the spleen after thyroid feeding, and in a later paper Speidel (1926*a*) claims that excess thyroid hormone causes a destruction of old red blood cells in the liver and a proliferation of young lymphoid haemoblasts. Speidel (1926*b*) also correlates the lymphoid hyperplasia associated with hyperthyroidism in man with that brought about in frog larvae by thyroid feeding. M. Kunde (1926) states that polycythemia may be produced in hypothyroid rabbits by oral administration of desiccated thyroid or by injection of thyroxin, although the haemoglobin content of the blood may remain below normal.

Isolated blood counts taken in clinics on hyperthyroid individuals have been contradictory. In a general review of the relations between the thyroid and the blood, Crotti (1922, p. 41) writes that in "hyperthyroidism the red corpuscles do not show any modification in quality nor in quantity" and "that the number of leucocytes is reduced, but hyperlymphocytosis combined with hypopolynucleosis is present." In hypothyroid conditions (either spontaneous or in thyroidectomized animals), the red cells drop and leucocytes rise,

[1] The data in this paper were presented by the junior author (F. S.) in partial fulfilment of the requirements for the degree of Master of Arts and were reported in abstract in the *Anatomical Record*, XXXVII (1927), 141. The expenses of the work were in part defrayed by a grant from the Society of Sigma Xi for the study of blood by the supravital method.

although later the latter may diminish in number. Mackenzie (1926) cites findings of Kocher and others similar to these except that at times the red-cell count may be slightly increased. On the other hand, hyperthyroidism may occasionally be characterized by an anemic condition (more often associated with hypothyroidism); and Baker (1924) explains his clinical findings of a decreased red-blood-cell count in some cases as the probable result of a high-grade intestinal toxemia brought about by the hyperthyroid condition. In these instances he also notes a low haemoglobin content of the blood, a lowered number of polymorphonucleated leucocytes, an increase in large lymphocytes, and a slight advance in the number of eosinophiles and monocytes. From an analysis of data secured in mild cases of hypothyroidism, Hutton (1924) believes that there is a definite relation between the percentage of polymorphonucleated leucocytes and the degree of activity of the thyroid gland. In 80 per cent of his hypothyroid cases the differential count showed a lowered number of polymorphonucleated cells. Zondek and Koehler (1926) report that after administration of 0.1–0.3 grams of thyroid—in some cases to healthy individuals, in others to sick persons—there usually is an increase, but there may be a decrease in red blood cells. A decrease in total white count followed thyroid administration in certain cases, while the differential white count was decidedly influenced in a case of Basedow's disease in that there was a decrease in lymphocytes and an increase in polymorphonucleated leucocytes. In three cases they also cite an increase in basophiles, eosinophiles, leucocytes with ring nuclei, and in only one instance an increase in monocytes. All of these data indicate that there is a reaction of the blood cells to the heightened metabolic condition in hyperthyroidism or the lowered one of hypothyroidism.

The present study was undertaken in the hope that it might be possible to determine more definitely the type and significance of the reaction of the corpuscles to the thyroid hormone by taking blood counts on consecutive days on rats in which experimental hyperthyroidism had been produced by feeding. During the course of the investigation it has been found that there is a rhythmic succession of increases and decreases in the total counts of the blood cells, accompanied by variations in form and altered distribution in

types of cells, and an attempt has been made to analyze these changes.

MATERIAL AND METHODS

Albino rats between the ages of twelve and sixteen months from the Wistar Institute Experimental Colony and between the ages of six months and one year from the stock of Mount Holyoke College have been used in these experiments. The blood was obtained from the tip of the tail by pricking with a Keith's abdominal needle after the surface had been gently rubbed with xylene to dilate the veins, cleaned with 80 per cent alcohol, and allowed to dry. The first drop of blood was always discarded. When a generous flow was not obtained, the tail was stroked from base to tip, or if necessary, a new hole was made.

TOTAL COUNTS

Standardized blood-counting pipettes and a Levy counting-chamber with double Neubauer rulings were used for both red and white blood counts. The blood was diluted 1:200 with a modified Hayem's solution for red blood corpuscles and 1:20 with 0.3 per cent acetic acid freshly made every day for white blood corpuscles, except when otherwise noted. Pipettes were shaken uniformly and counts were made from two different drops. An area of 0.4 sq. mm. was counted for red blood cells and an area of 8 sq. mm. for white blood cells.

DIFFERENTIAL COUNTS

Supravital technique.—The supravital technique followed was that of Sabin as described by Cunningham, Sabin, Sugiyama, and Kindwall (1925). Variations in dilutions were made to compensate for the differences in stain, i.e., post-war instead of pre-war Grübler's neutral red, and for changes in blood-cell concentration. The staining solution used was 0.6 cc. of a 1 per cent solution of neutral red in absolute alcohol to 10 cc. of absolute alcohol plus five drops of saturated solution of Janus green in absolute alcohol. Fresh Janus green solution was made each time and mixed with neutral red just before using. A mixture of three parts of paraffin to one part of vaseline was used to seal cover glasses to the slides.

Fixed supravital smears.—It seemed advantageous to preserve the staining reaction of the living blood cells to neutral red and

Janus green. For this purpose a clean slide was prepared with transverse bands of paraffin about $\frac{1}{4}$ inch from each end of the slide. A blood smear was made on a slide with a film of neutral red and Janus green (Sabin 1923), covered by the slide described above, and sealed with paraffin. The paraffin bands were thick enough to prevent the slide from touching the blood smear but not so thick that there was danger of drying from too large an air chamber. The slides were then placed in a warm box[1] for 15 or 20 minutes, after which the blood smear was uncovered and placed, film side up, over 40 per cent formaldehyde in a Syracuse dish within a moist chamber; in this way the cells were fixed by the formaldehyde fumes within 1 or 2 minutes. It was found that these slides were more satisfactory if covered with balsam.

Fixed smears.—Fixed blood smears were made by the slide method and stained in Wright's stain (National Stain and Reagent Company). Two hundred cells were counted in each smear.

Reticulated red-blood-cell counts.—A large drop of blood was placed on a clean flamed slide, and a small drop of 1 per cent brilliant cresyl blue in normal salt solution was then run into the drop of blood from a pipette. The mixture of blood and stain was smeared on the slide by gently drawing a cover slip over it. A cover slip was then placed over the smear and sealed down with the paraffin and vaseline mixture. In making the counts, an oil immersion lens and an ocular with ruled squares were used. One thousand red blood cells were counted within the ruled area to obtain the percentage of reticulated cells.

Haemoglobin test.—The test for haemoglobin was made with Tallquist's haemoglobin scale.

Measurements.—Cells were measured with an ocular micrometer.

NORMAL BLOOD OF ALBINO RAT

The somewhat contradictory statements of observers concerning the blood count and the blood picture of albino rats, as well as the lack of counting done on rat blood with the supravital method, made necessary a study of normal blood before the study of the blood of

[1] The pattern for this warm box was kindly given to the Zoölogy Department by Dr. Florence Sabin, of the Rockefeller Institute.

TABLE I

COMPILATION OF BLOOD COUNTS ON ALBINO RATS (148 OR MORE DAYS OLD)

(Data from Donaldson [1924, pp. 68–75], Addison and Richter [1924], Falconer [1926], and Authors)

Name of Investigator	No. of Cases and Sex	Weight in Grams	Age in Days	Percentage of Haemoglobin	Red Cells		White Cells									
					Millions in 1 Cu. Mm.	Percentage of Reticulated Cells	Cells in 1 Cu. Mm.	Polymorphonuclear Leucocytes %			Lymphocytes %		Monocytes %		Endothelial Cells %	Unclassified %
								Neut.	Eos.	Bas.	S.L.	L.L.		Tr.		
Jolly, 1906, 1909				?365		8–10		10,000								
Rivas, MS, 1914	3(W)?			full-grown	86.0	8.7		8,133	57.0	1.12	0.28	34.60	7.00			
	3(W)?			full-grown	90.0	7.9		8,467	52.5	0.60	0.30	41.70	4.90			
	4(W)?			full-grown	96.0	8.0		11,450	55.9	0.90	0.20	38.20	4.80			
Margot, MS, 1916	3(W)♂	233	148		103.0	9.4		9,375	59.0	0.80	0.00	33.80	6.30			
Nowrey, MS, 1921	11(W)♂	267	163					9,281	62.07	1.54	0.42	29.55	2.45	1.83	0.77	1.37
	12(W)♀	179	165					8,555	55.71	4.00	0.45	30.92	3.56	2.49	1.20	1.67
Cramer, Drew, Mottram, 1921, 1922	5	110–150				10.2–10.9			% value ½ that of S.L.*			% value 2× that of Neut.*				
Addison, Richter, 1924			150 or mature			7.5–9.0		8,000 or more	10–25							
Falconer, 1926	10 (13 counts)	200–320				Av 9.22 Mx 9.76 Mn 8.20		Av 10,291 Mx 16,700 Mn 6,640	Av 24.8	Av 7.3		Av 56.0		Av 14.0		"Unknown cells"
Authors (present paper)	4(M)♀ (13 counts)	Av 220	180–360	Av 96.0 Mx 100.0 Mn 90.0	Av 9.07 Mx 10.04 Mn 8.17		Av 10,750	Av 12.1	Av 2.0	Av 0.40	Av 49.6		Av 3.7		Av 2.7	Av 30.2
	2(M)♂ (14 counts)	Av 231	180–360	Av 95.0 Mx100.0+ Mn 90.0	Av 9.53 Mx 10.37 Mn 8.13	Av 3.2† Mx 5.1 Mn 2.3	Av 9,997	Av 24.7	Av 2.2	Av 0.75	Av 44.8		Av 2.9		Av 0.95	Av 17.8
	6(W)♀ (18 counts)	Av 232	360–480	Av 95.6 Mx 100.0 Mn 90.0	Av 9.26 Mx 11.43 Mn 7.95	Av 1.86 Mx 3.20 Mn 1.00	Av 12,540 Mx 17,275 Mn 7,825	Av 33.5 Mx 40.0 Mn 23.0	Av 2.8 Mx 8.0 Mn 1.0	Av 0.02 Mx 1.00 Mn 0.00	Av 45.4 Mx 52.0 Mn 35.5		Av 1.2 Mx 4.0 Mn 0.0		Av 2.0 Mx 7.0 Mn 0.0	Av 12.9 Mx 28.0 Mn 5.0
	4(W)♂ (15 counts)	Av 274	360–480	Av 97.7 Mx 100.0 Mn 95.0	Av 9.92 Mx 10.95 Mn 9.58	Av 2.74 Mx 3.90 Mn 2.00	Av 13,379 Mx 19,200 Mn 8,225	Av 31.1 Mx 41.0 Mn 24.0	Av 1.8 Mx 6.0 Mn 0.5	Av 0.08 Mx 2.00 Mn 0.00	Av 50.0 Mx 59.0 Mn 27.0		Av 1.56 Mx 4.00 Mn 0.00		Av 0.8 Mx 3.5 Mn 0.0	Av 15.0 Mx 38.0 Mn 5.0

W = Wistar, M = Mount Holyoke stock; Av = Average, Mx = Maximum, Mn = Minimum, Neut. = Neutrophile, Eos. = Eosinophile, Bas. = Basophile, Tr. = Transitional.

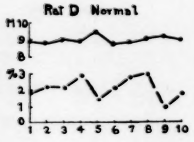
* These values agree with those of Kleineberger and Carl (1912), Eyre (1913), and some other authors (names not given) cited by Donaldson (1924, p. 74–75).

† One rat, 9 counts.

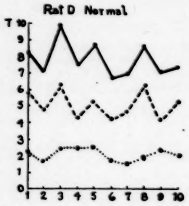
thyroid-fed rats was undertaken. The diversity of observations is evident in Table I, which presents a compilation of the determinations by various authors as given in Donaldson's book, *The Rat* (1924, pp. 68–75), as well as data from Addison and Richter (1924), Falconer (1926), and those secured in the present study.

RED BLOOD CELLS

Size, shape, number.—The majority of the red blood cells of albino rats are non-nucleated biconcave disks with an average di-



1*a*



1*b*

FIG. 1*a*, *b*.—Curves showing variation in red and white counts in a normal albino rat (rat D), Mount Holyoke stock, over 10 consecutive days. Fig. 1*a*————, total red count; —— —— ——, percentage of reticulated cells. Days are given on abscissa, millions (M) of cells per cu. mm. and percentage of reticulated cells on ordinate. Fig. 1*b*:————, total white-cell count; — — — — —, lymphocytes; · · · · · · · · · ·, leucocytes. Days are given on abscissa, thousands (T) of white cells, lymphocytes, and leucocytes per cu. mm. on ordinate.

ameter of 6.86 micra.[1] The total red blood count as observed in the two strains studied is about the same. In rats from the Wistar Institute colony the average total red count for the male (9,921,661) is slightly higher than that for the female (9,262,314), and a similar difference exists in the two sexes of rats from the Mount Holyoke colony: male, 9,530,000; female, 9,070,000 (Table I). The range of variation occurring in 10 consecutive days is relatively slight, as may be seen in Figure 1*a* (rat D, Mount Holyoke stock).

Reticulated red blood cells.—In addition to the typical non-nucleated erythyrocytes, some of the red cells in normal rats contain varying amounts of basophilic substance in the form of reticulations. The percentage of reticulated cells varies in individual rats and on

[1] According to Donaldson (1924, p. 71), measurements given by other authors are as follows: Jolly (1919), 6.6 μ at 90 days; Treadwell, 6.5 μ; Wormley (1888), 7 μ; Gulliver (1875), 6.5 μ.

consecutive days (Table I and Fig. 1), the maximum observed in normal animals being 5.1 per cent, the minimum 1 per cent. The average for 9 rats (42 counts) is 2.46 per cent. These reticulations stain a deep purplish blue in brilliant cresyl blue, while the cytoplasm of the corpuscle does not react to the stain. The reaction to neutral red (Plate I, Fig. 2) is less sensitive than to cresyl blue and gives a light to a deep brick-red color, which in old slides often appears brownish. Reticulations do not react to Wright's stain, but a corresponding polychromasia is present.

The amount of reticulation in different cells varies from a few round bodies or granular precipitations on scattered threads to masses clumped at the center or throughout the cell. Occasionally in pathological blood containing a high percentage of reticulated cells a heavy, wreathlike formation is found around a relatively clear area. Probably this corresponds to what Doan, Cunningham, and Sabin (1925) call the "third stage" of reticulation in the developing chick erythrocyte and from which they consider the true reticulations to arise. Hereafter this cell will be referred to as Stage 1, and the fainter and later stages of reticulations will be called Stages 2, 3, 4, and 5 (Plate I, Figs. 3–7). In the normal blood of albino rats the stages most frequently seen are 5 and 4, although a few cells of Stage 3 and an occasional one of Stage 2 have been observed.

Sometimes a refractive body (Plate I, Fig. 2) is seen in some or most of the cells of certain smears. This body does not react to neutral red, Janus green, brilliant cresyl blue, or to Wright's stain. It does not correspond to the descriptions of the refractive-granule red blood corpuscle or the Howell body as described by Isaacs (1925). While this refractive body may be found in almost all of the cells of one slide, none may be seen on a similar slide made at the same time from the blood of the same animal. Since it may be an artifact or a degeneration phenomenon due to technique, cells containing it have not been included in reticulated red-blood-cell counts.

Percentage of haemoglobin.—The percentage of haemoglobin in the normal blood of the two stocks used varied from a minimum of 90 to a maximum of 100, with an average for the Mount Holyoke stock (both sexes) of 95.5 per cent and for the Wistar stock (both sexes) of 96.5 per cent (Table I).

PLATE I

(The authors wish to express their gratitude to Margaret Chambers who redrew the figures on Plate I from originals in color.)

FIGS. 2–7.—Reticulated red blood cells of rat from preparations supravitally stained with neutral red (Fig. 2) and brilliant cresyl blue (Figs. 3–7). Cells in Figures 2 and 3 are from blood of rat fed 4 grains of thyroid daily for 10 days; those in Figures 4–7 are from normal blood.

FIG. 2.—Group of cells showing variation in reticulations. Sizes relative; *a*, cell with refractive body.

FIG. 3.—Stage 1. Cell diameter 7 μ. $\times$2,133 (approx.).

FIG. 4.—Stage 2. Cell diameter 6.5 μ. $\times$2,222 (approx.).

FIG. 5.—Stage 3. Cell diameter 7.6 μ. $\times$2,303 (approx.).

FIG. 6.—Stage 4. Cell diameter 7 μ. $\times$2,133 (approx.).

FIG. 7.—Stage 5. Cell diameter 7 μ. $\times$2,133 (approx.).

FIG. 8.—Neutrophile with "ring nucleus" and large vacuoles. From preparation supravitally stained with neutral red and Janus green. (Darker spots are mitochondria.) Cell dimensions: 12.2 μ $\times$ 11.6 μ $\times$ 16 μ. $\times$2,500.

FIGS. 9–11.—"Unknown cells," from preparations supravitally stained with neutral red and Janus green.

FIG. 9.—Cell containing neutral red granules, mitochondria (darker spots), but lacking vacuoles. $\times$2,500.

FIG. 10.—Cell containing vacuoles stained with neutral red. Mitochondria not evident. $\times$2,500.

FIG. 11.—Cell drawn in active motion. Arrow shows direction of movement. $\times$2,500.

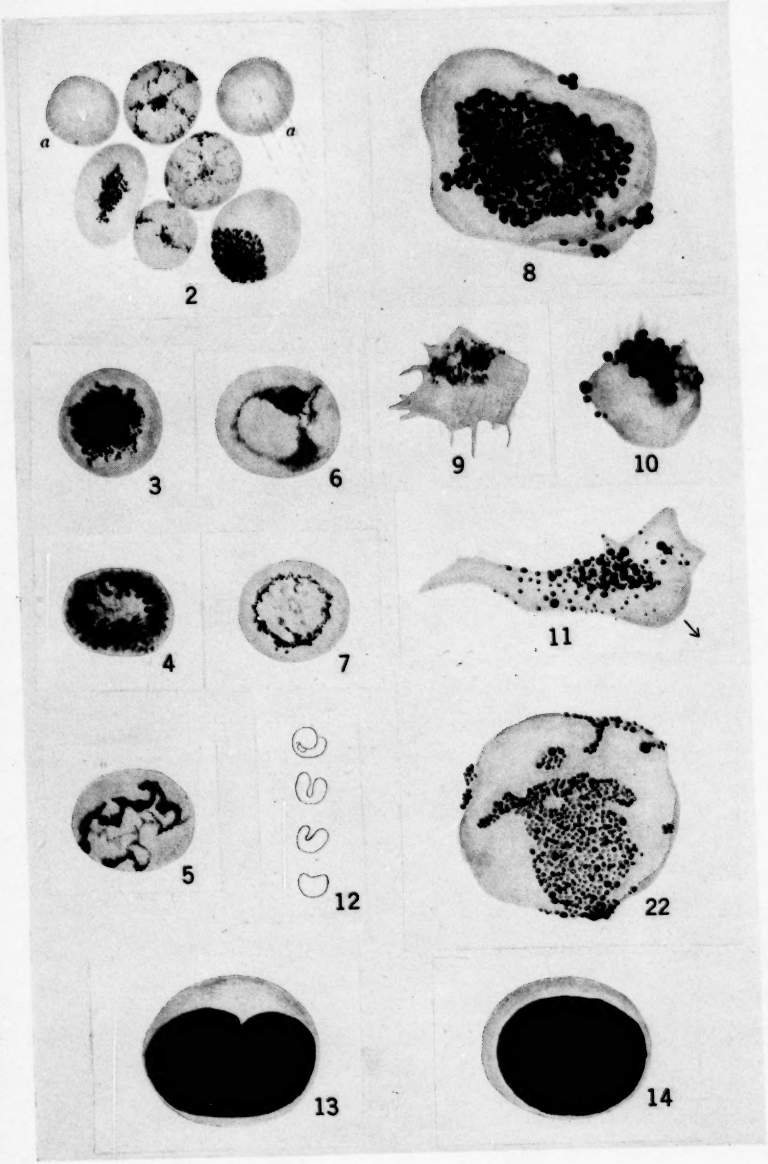
FIG. 12.—Diagram showing variations in shape of nucleus of "unknown cell."

FIG. 13.—"Unknown cell" drawn from fixed smear, Wright's stain. For relative size, compare with Figures 9–11.

FIG. 14.—Small lymphocyte drawn from fixed smear, Wright's stain.

FIG. 22.—Late myelocyte supravitally stained with neutral red and Janus green. (Darker spots are mitochondria.) Cell diameter, 14.4 μ. $\times$2,322 (approx.).

PLATE I



WHITE BLOOD CELLS

Types.—Eight types have been found among the white blood cells of the albino rat. These are the polymorphonucleated neutrophile, eosinophile, basophile, small and large lymphocyte, monocyte, endothelial cell, and an "unknown cell." Of these the eosinophiles, basophiles, small and large lymphocytes, and monocytes are similar in all respects to the corresponding cells in the human and rabbit blood described by Sabin (1923), while the endothelial cells resemble those of the rabbit observed by Sabin and Doan (1926). The neutrophilic leucocyte and the "unknown cell" deserve special consideration.

The neutrophilic leucocytes of the albino rat have two types of nuclei, the typical polymorphic nucleus and the ring nucleus. Cells with the former are similar to human and rabbit cells of the same type (Sabin 1923), although the vacuoles seem to be more numerous. The cells with the ring nucleus (Plate I, Fig. 8) differ from the polymorphic type in the shape of the nucleus and in the presence of mitochondria, since, according to Cunningham, Sabin, and Doan (1925), practically no mitochondria are found in the very late myelocyte. They comprise from 10 to 40 per cent of the total leucocyte count.

The "unknown cell" (Plate I, Figs. 9–13) has been studied in some detail and compared with the other types of white blood cells in an effort to classify it with one of the major groups. Its chief characteristics are an average diameter of 9.2 μ (for 50 cells)—some of the cells range from 7–13 μ—a cell membrane which is never well defined, a spherical shape except when in motion when it extends small, clear, tonguelike pseudopodia (Plate I, Fig. 11), an excentric, slightly mottled nucleus which is commonly bean-shaped and averages about one-half or three-quarters of the size of the cell (Plate I, Figs. 12, 13). This nucleus is always carried at the advancing end of the cell with the *Hof* facing backward when the cell is in motion (Plate I, Fig. 11), in contrast to the movement in the monocyte where the nucleus appears simply to be carried along by the cell. The rate of movement of the "unknown cell" is also slower than that of the monocyte or polymorphonucleated neutrophile. In the cytoplasm which has a "ground-glass" appearance are found neutral red gran-

ules (Plate I, Fig. 9) of unequal size and staining capacity, which may or may not be arranged in a rosette around the centrosphere in the *Hof* of the nucleus. The mitochondria (Plate I, Fig. 9) are scattered throughout the cytoplasm without any definite arrangement, and sometimes are not evident (possibly they are obscured by the abundance of vacuoles or are absent due to age). The vacuoles (Plate I, Fig. 10) are of various sizes—the larger ones may be 3 or 4 μ in diameter—and are usually found in the cytoplasm opposite to and in the *Hof* of the nucleus and may be scattered among the granules forming the rosette, while in the monocyte they, with the mitochondria, consistently form a wreath about the rosette (Cunningham, Sabin, Sugiyama, and Kindwall, 1925). These "unknown cells" may ingest red cells but can be distinguished from desquamated endothelial cells by the unfailing presence of mitochondria in the "unknown cells" which show phagocytic activity.

After carefully comparing the "unknown cell" with the monocyte and the lymphocytes of the blood stream,[1] the cell was grouped in normal and experimental data with the lymphocytes chiefly because of the following likenesses to the latter: (1) the mode of locomotion in both of the cells by means of blunt, clear pseudopodia with the nucleus carried at the advancing end of the cell; (2) the mottled appearance of the nucleus (cf. Plate I, Figs. 13, 14); (3) the distribution of mitochondria throughout the cell; (4) the vacuoles which are more numerous in the "unknown cells" than in the lymphocytes, although, according to Sabin (1925), lymphocytes may have a number of vacuoles under certain stimulating circumstances (possibly these stimulating factors are normally present in the blood of the rat); (5) a direct correlation in the number of lymphocytes and of "unknown cells" in pathological blood so that an increase in

[1] In an attempt to see if the "unknown cell" might be of the same origin as the lymphocyte, smears were made of lymph nodes supravitally stained with neutral red and Janus green from five albino rats. In one smear only were there many cells containing vacuoles at one side of the cell. In these the nucleus was the same as that of the "unknown cell," but it lacked all of the other characteristics of the "unknown cell" and of the monocyte. A study was also made of two mesenteric lymph nodes of another rat. These nodes were supravitally stained and fixed according to McJunkin's technique (1925) and sections 10 μ in thickness were cut. In this case the reaction to stain of all cells of the lymph nodes except the fibroblast was negative. Further work needs to be done on this phase of the problem before any conclusion can be drawn.

lymphocytes is always associated with an increase in the "unknown cells."[1]

The total and differential white counts vary somewhat in the two strains of rats used (Table I) and also in the sexes. The average total count for males of the Wistar stock is 13,379 cells; in the Mount Holyoke strain, 9,997. In the case of females of the former, the average is 12,540 cells; in the latter, 10,750. The average percentage of leucocytes in the Wistar Institute stock is also higher, being 34.65 per cent as compared with 20.9 per cent in the Mount Holyoke strain. If the "unknown cells" are considered with the lymphocytes, the average percentage of lymphocytes in the blood of the Wistar strain becomes 61.65 per cent and in the blood of the Mount Holyoke stock 71.2 per cent. If a comparison be made in a general way between the results of previous investigators and those presented here (Table I), it will be seen that in the former, with few exceptions,[2] the percentage of lymphocytes is about one-half that of the polymorphonuclear neutrophiles, while in the latter and in the exceptions noted the reverse is true. The variations in the total and differential white count in a normal rat (rat D, Mount Holyoke stock) over 10 consecutive days may be seen in Figure 1*b*.

BLOOD OF THYROID-FED RATS

EXPERIMENTAL PROCEDURE

Twelve rats (seven females, rats A, D, E, F, J, P, N, and five males, rats B, G, H, L, O) were fed daily on thyroid in amounts varying from ½ to 8 grains, Table II. Each animal was weighed before thyroid administration. Four additional animals (rats K, M, R, S) were splenectomized, and each was fed 4 grains of thyroid daily over a period of time after the operation to see the reaction of the red cells to this procedure and compared with a fifth (rat Q) fed thyroid simultaneously but not splenectomized. Desiccated thyroid

[1] If "unknown cells" are included with the monocyte group, it makes a percentage of monocytes much higher than that recorded for any known form of mammalian blood. Compare, however, Falconer (1926), Table I, in which it seems probable that the "unknown cells" are classified in the monocyte group.

[2] The exceptions listed in Donaldson (1924) are Kleineberger and Carl (1912); Eyre (1913); Cramer, Drew, and Mottram (1921); Mottram (1922). Falconer (1926) would also be an exception.

TABLE II

THYROID FEEDING EXPERIMENTS—ALBINO RATS

Rat and Sex	Stock*	Age in Mos.	Weight in Grams at Start of Experiment	Amount of Thyroid Fed Daily (Grains)	No. of Counts before Thyroid Feeding	No. of Counts on Consecutive Days after Thyroid Feeding	No. of Counts at 4- or 5-Day Intervals after Thyroid Feeding	No. of Days after Thyroid Feeding Covered by Counts on Consecutive Days or at 4- or 5-Day Intervals†
A♀	M	6–12	219.0	½	2	10	7 (28 days)	38
B♂	M	6–12	230.0	½	2	8	7 (28 days)	36
D♀	M	6–12	219.0	1	10	7	5 (20 days)	27
E♀	W	12–16	257.0	2	2	10	9 (43 days)	53
F♀	W	12–16	235.5	2	2	10	9 (43 days)	53
G♂	W	12–16	281.9	2	2	10	9 (43 days)	53
H♂	W	12–16	294.5	2	2	10	1 (4 days)	14
J♀	W	12–16	196.2	4	2	10	3 (12 days)	22
L♂	W	12–16	285.4	4	2	9	2 (8 days)	18
P♀	W	12–16	240.0	4	2	10	1 (4 days)	14
N♀	M	6	228.5	8	2	10	1 (4 days)	14
O♂	M	6	232.8	8	2	10	1 (4 days)	14
X?	W	12–16	?	0	10	.....	.....	.....

* M = Mount Holyoke stock; W = Wistar stock.

† In some cases rats were fed after counts at 4- or 5-day intervals were discontinued, and isolated counts were made at various times. See text, p. 196.

tablets (Armour and Company) containing 0.2 per cent iodine were ground to a fine powder, mixed with milk and whole-wheat graham flour to make a thin paste, and administered orally but separately from the regular diet. From the first this mixture was greedily swallowed by the animals. Daily thyroid feeding in the amounts indicated in Table II was continued until the animal died or the experiment was discontinued.

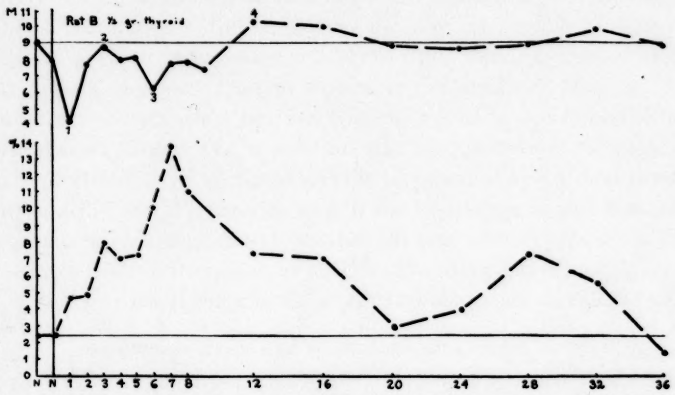


FIG. 15.—Curves showing variation in red count ————, and percentage of reticulated cells — — —, in rat B, fed ½ grain of thyroid daily. Period of counts at 4- or 5-day intervals indicated by a change in style of line. Days are given on the abscissa (N, normal day preceding thyroid feeding; 1 ff., days after thyroid feeding); millions (M) of red cells per cu. mm. and percentage of reticulated cells on ordinate. Lines parallel to abscissa are intended as guide lines for normal levels. Numbers 1, 2, 3, 4, on total red curve indicate points mentioned in text. Consecutive daily counts end on eighth day.

Complete blood counts were made on these animals at regular intervals after thyroid administration. The term "complete count" as used in this paper indicates total white, total red, red reticulated, supravital differential, fixed differential counts, and haemoglobin determination. As far as possible, the counting was done at the same time on consecutive days during the experiment. After the counts made over the 10 consecutive days, counts were made every 4 or 5 days until the death of the animal or the discontinuation of the experiment. On the two days immediately preceding thyroid

feeding, complete counts were made on each experimental animal which were considered the normal control counts; and in addition the series of 10-day counts made on each of two normal rats, rat D from the Mount Holyoke strain (Figs. 1*a*, *b*) and rat X from the Wistar stock, served to give an idea of the normal range of variation in the two stocks.

The general blood reaction of rats of both sexes in the two strains and of different weights to the thyroid feeding is the same, and therefore curves plotted for red-cell counts resemble each other fundamentally even though the counts are made after varying dosages ($\frac{1}{2}$–8 grains). Similarly a uniformity of pattern exists among the white-cell curves, although in both red and white curves variations in degree of raction appear. In the case of the 8-grain dosage, the general bodily reaction was so severe, resulting in indigestion, diarrhea, and loss of appetite, that it was necessary to discontinue this part of the experiment, and the data are not included in the averaged curve although the counts made over 10 consecutive days formed a curve similar in essentials to those after smaller doses.

EFFECTS OF THYROID FEEDING ON RED BLOOD CORPUSCLES

(Rats A, B, fed $\frac{1}{2}$ grain; rat D, 1 grain; rats E, F, G, H, 2 grains; rats J, L, P, 4 grains thyroid daily. Figs. 15, 16, 17, 18.[1])

Changes in total red-blood-cell count.—Four main changes (Figs. 15, 16, 17, 18) due to thyroid feeding were observed in the numbers of red cells. (1) There is a rapid drop in total red-blood-cell count within 2–5 days for all animals, which in some cases reaches a maximum of 3,000,000 or more below normal. (2) Following this drop, a rise occurs which almost brings the count to the normal level within 1 or 2 days after the drop in the animals fed $\frac{1}{2}$ and 1 grain of thyroid (Fig. 15, rat B); while in the animals given 2 grains (Fig. 16, rat E) or 4 grains (Fig. 17, rat L) daily, the rise is slower and usually remains considerably below the normal. (3) This increase is succeeded by a general decrease in the number of red blood cells. Occasionally there are small irregularities in the dropping curve, but the slope is consistently downward. Within the first 6 or 7 days after the first

[1] The curves in Figures 15, 16, 17, 19, 20, and 21, are representative of the reaction in blood picture following thyroid feeding; while Figure 18 gives the results of averaging the data at corresponding points (see text) in animals given doses of $\frac{1}{2}$, 1, 2, and 4 grains of thyroid daily.

count in rats given ½ grain (Fig. 15) and 1 grain daily and within 6–10 days in the ones fed 2 (Fig. 16) and 4 (Fig. 17) grains, the red-

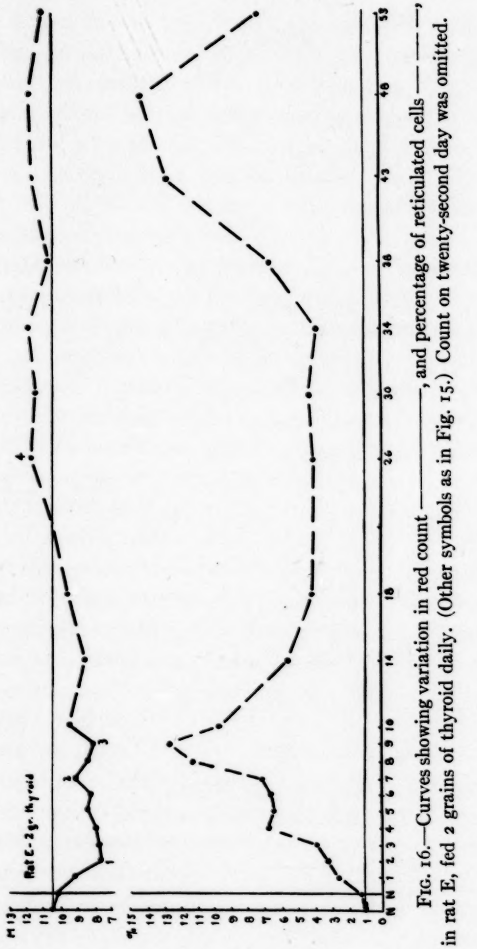


FIG. 16.—Curves showing variation in red count ————, and percentage of reticulated cells — — —, in rat E, fed 2 grains of thyroid daily. (Other symbols as in Fig. 15.) Count on twenty-second day was omitted.

cell determination is from 2,000,000 to 5,000,000 below normal. (4) This second fall is followed by a gradual rise, which may be uneven with small fluctuations; but the tendency is such that within 12–26

days[1] after the beginning of thyroid feeding, depending on the individual animal, the total red-cell count reaches a normal point or above, which it tends to maintain with some slight variations.[2] In rats E and F (fed 2 grains of thyroid daily) the total count at the thirty-fourth day was respectively 1.3 and 1.2 millions above normal; while in these same rats at the forty-eighth day, E gave a total red count of 1.5 and F of 3.1 millions above normal. In rat A likewise, the total count of 12,700,000 cells made 3 months after the beginning of thyroid feeding is 3,700,000 cells in excess of the original normal count. It is interesting to compare the curves for the 10-consecutive-day periods with the curve showing the normal condition (Fig. 1*a*) and to note the marked difference in pattern. The smoothed curve in Figure 18 made from averaged data of the thyroid-fed animals also brings out the general nature of the reaction and makes the fluctuations more apparent.

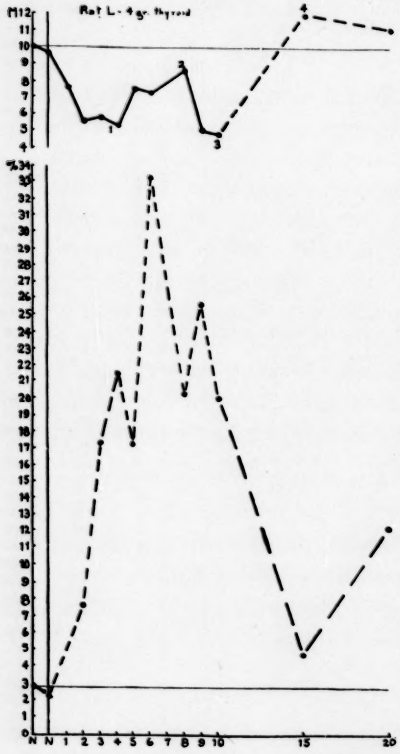


FIG. 17.—Curves showing variation in red count ————, and percentage of reticulated cells — — —, in rat L, fed 4 grains of thyroid daily. (Other symbols as in Fig. 15.) Count on seventh day was omitted.

[1] Since this point falls within the period when counts are being made at 4- or 5-day intervals, it may possibly have occurred before (or occur after) the day on which it appears in the records.

[2] In Figure 15 (rat B), the curve lies within or only slightly above the normal limits up to the thirty-sixth day; but in a count made 3 months after the beginning, a distinct polycythemia was evident, as in rat A at the same time and on the same dosage.

Changes in reticulated red-blood-cell count.—Corresponding to the drop in the total number of red blood cells, there is a rise in the number of cells with reticulations (Figs 15, 16, 17, 18). The increase in each animal is uneven and usually reaches its highest point before or during the second rise in total red count. The percentage of reticulations in rats fed 4 grains of thyroid is markedly great (Fig. 17, rat L, 33 per cent above normal). A steady decrease in the reticulated cells correlates with the increase in the total number of reds. In animals in which the counts are continued over more than 15 days (at 4- or 5-day intervals), a second less-marked rise in reticulations takes place (Figs. 15, 16, 17).

Changes in the microscopic appearance of the red blood cells.—Corresponding to the increase in the reticulations which are seen in the supravitally-stained red cells, there is marked evidence of polychromasia in fixed smears colored with Wright's stain. Occasionally, nucleated red cells are also present, but the number is so small that it is not feasible to calculate the percentage. Throughout the period of high reticulations a mild anisocytosis caused by a large number of young cells is evident in fixed and stained smears. Otherwise the cells are normal. No poikilocytosis accompanies the anisocytosis. Directly proportional to the increase in percentage of reticulated cells is the increase in the amount of basophilic substance in each cell. Stages 4 and 5 (Plate I, Figs. 6, 7), which are typical of normal blood and are present at the beginning of the rise, are replaced by an abundance of Stages 2 and 3 (Plate I, Figs. 4, 5) at the time of the greatest number of reticulated cells. This reaction seems to be more marked in rats fed the higher dosages of thyroid, especially in those receiving 4 grains daily.

Changes in the haemoglobin content.—There is a lessening of the haemoglobin content of the blood corresponding to the fall in red blood cells. Although the haemoglobin rises slowly after its initial drop, its ratio to the number of red blood cells remains slightly lower than that of normal blood throughout the duration of the experiment.

EFFECTS OF THYROID FEEDING ON WHITE BLOOD CORPUSCLES

(Rats A, B, fed ½ grain; rat D, 1 grain; rats E, F, G, H, 2 grains; rats J, L, P, 4 grains thyroid daily; Figs. 18, 19, 20, 21.[1])

[1] See footnote 1, p. 194.

Changes in total white-cell count.—The total white-cell count is affected by thyroid feeding, the higher dosages (2 and 4 grains daily)

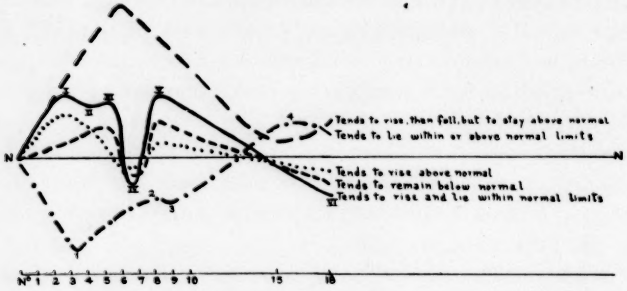


FIG. 18.—Smoothed curves made from averages of data at corresponding points (e.g., Points I, II, etc.), showing the correlation between variations in total red count ·—·—·, percentage of reticulated cells — — —, total white count ————, lymphocytes – – – – –, leucocytes · · · · · ·, after feeding of ¼, 1, 2, or 4 grains of thyroid daily to rats. Days indicated on base line. Points 1, 2, 3, 4 are marked on total red count curve; Points I, II, III, IV, V, VI, on total white-count curve. All points except VI based on data from ten rats, VI on eight rats. Line from N parallel to base line represents normal level.

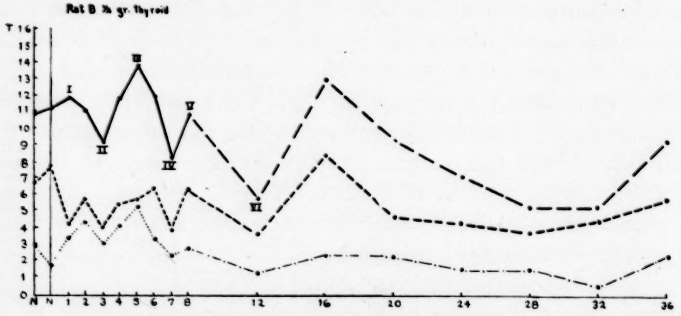


FIG. 19.—Curves showing variation in total white count ————, lymphocytes — — — — —, leucocytes · · · · · ·, in rat B, fed ½ grain of thyroid daily. Period of count at 4- or 5-day intervals indicated by change in style of line. Days are given on abscissa, thousands (T) of white cells, lymphocytes, and leucocytes per cu. mm. on ordinate. Numbers I, II, III, IV, V, VI, on total-count curve represent points mentioned in text. Consecutive daily counts end on eighth day.

having the most marked effect. The cells which are responsible for the changes in total count are the neutrophilic leucocytes and the

lymphocytes, there being no marked difference in the eosinophiles (cf. p. 202), basophiles, monocytes, or endothelial cells throughout

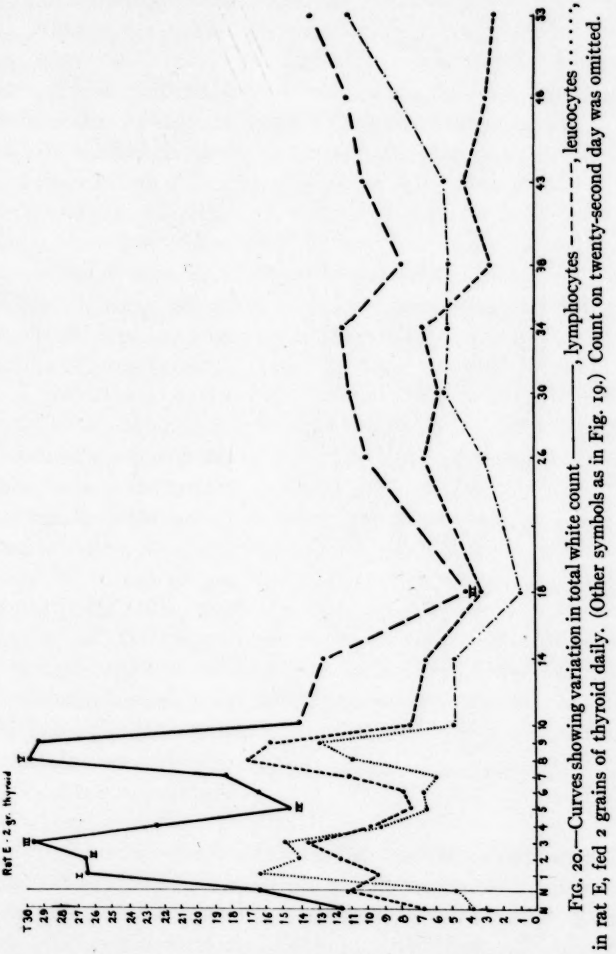


FIG. 20.—Curves showing variation in total white count ————, lymphocytes – – – – –, leucocytes . . . . . , in rat E, fed 2 grains of thyroid daily. (Other symbols as in Fig. 19.) Count on twenty-second day was omitted.

the experiment. In a few of the animals a drop of varying extent below normal in total number of white cells occurs in the first count after thyroid administration, but this does not appear to be a funda-

mental change. The first marked change (Point I, Figs. 18, 19, 20, 21) is a rapid high rise which occurs within 1–4 days after thyroid feeding and in which the counts range from 1,100 to 24,200 cells above the normal. The averages of the number of cells above normal according to dosages are: ½ grains (2 rats), 3,175 cells; 1 grain (1 rat), 4,050 cells; 2 grains (4 rats), 8,562 cells; 4 grains (3 rats), 17,300 cells. This rise is mainly due to the leucocytes (Figs. 18, 19, 20, 21) which consistently lie above their normal level although the lymphocytes are in general on the increase and tend to lie above their normal limits or within them. Following this rise appears a variation in total count (Point II) in which the curve tends to drop,[1] although as a rule it remains above the normal level unless the rise has been very slight, as in rat B (Fig. 19). A falling in the leucocytic number appears to be responsible for this change, as the lymphocytes with few exceptions (rat E, Fig. 20; rats G and L, Fig. 21) seem to be rising. The continued increase in numbers of lymphocytes associated with the continued dropping in the leucocytes brings about a

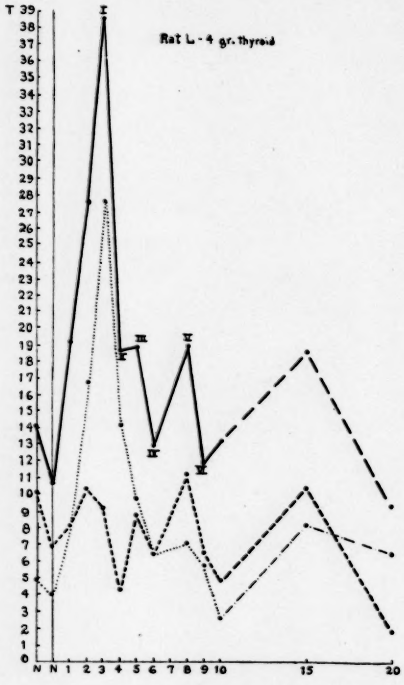


FIG. 21.—Curves showing variation in total white count ————, lymphocytes – – – – – –, leucocytes · · · · · ·, in rat L, fed 4 grains of thyroid daily. (Other symbols as in Fig. 19.) Count on seventh day was omitted.

[1] This drop did not occur in rat E (Fig. 20), but the cells tended to rise slightly above the level of Point I. It appears from the differential count, however, that this corresponds to the Point II in other animals.

second high point (Point III) within 3–8 days. The height of this point is influenced especially by the rate of decrease of the leucocytes, but in six of the ten animals it lies slightly above the first point. In the curve (Fig. 18) made from averaged data, however, it lies below Point I largely because in 2- and 4-grain animals the first reaction is so great that the second rise does not mount as high as the first one (cf. Fig. 21, rat L). Following the second high point, a drop (Point IV) occurs which is usually of marked extent and in nine of the ten rats shifts the total count into (Figs. 20, 21) or below (Fig. 19) the normal limits. A continued decrease in leucocytes, together with a greater dropping of lymphocytes, appears to be responsible for this change, which occurs within 5–9 days. A third rise (Point V) now takes place, which in general does not reach the height of the first two high points (I and III) and is more marked in rats fed 2 (Fig. 20) and 4 (Fig. 21) grains of thyroid. Increased numbers of both leucocytes and lymphocytes, but mainly of the latter, are responsible for this rise which appears during the last 5 days of the 10-day period, usually on the eighth or tenth day (Figs. 18, 19, 20, 21).

After the counts on 10 consecutive days when determinations are made every 4 or 5 days apart, it is more difficult to analyze the curves, although a drop (Point VI) well below normal, due to decrease in both leucocytes and lymphocytes, consistently follows the third rise (Point V) in each of the ten animals.[1] After this drop, the curves made from the data taken at longer intervals show irregularities in outline in total white and differential counts, but the tendency of the total count is to rise until it lies within the normal limits of variation, sometimes slightly above or slightly below, while the lymphocytes stay below their normal level (lymphopenia) and the leucocytes remain above the normal (leucocytosis).

Changes in the differential white-cell count.—Myelocytes (Plate I, Fig. 22) appear in the blood during the second to the fourth days after thyroid feeding, but their occurrence is neither consistent nor regular, being found during both high and low leucocyte counts. The highest percentage of myelocytes recorded in rats fed $\frac{1}{2}$ and 1 grain of thyroid is 4, while in those fed 2 and 4 grains, it reached 7

[1] See footnote 1, p. 196.

per cent. Eosinophiles increase slightly during the first high polymorphonuclear leucocyte count, and the increase is proportional to the increase in neutrophiles, so that the ratio of normal number of neutrophiles to the high number of neutrophiles is similar to the ratio of normal number of eosinophiles to the high number of eosinophiles. There is a gradual steady decrease in typical polymorphonuclear cells, which is more marked in blood of rats fed 2 and 4 grains of thyroid. In counts made on blood of these rats after 1½ months of daily thyroid feeding, 90–95 per cent (as compared with the normal 10–40 per cent) of the neutrophiles have ring nuclei and contain a large number of mitochondria in the form of small granules or fairly large droplets. Occasionally a centrosphere is seen in these cells. No changes are observed in the basophiles. Both large and small lymphocytes are slightly more active. There is also a proportional increase or decrease in number and activity of the "unknown cells" corresponding to those of the lymphocytes. The non-motile cells which are not too degenerate to identify are counted with their own types. In counts containing a high percentage of non-motile cells, the cause was always traced back to mechanical injury.

DISCUSSION

The data from the blood counts of thyroid-treated rats indicate that the blood picture is affected by hyperthyroidism and therefore give indirect evidence that the blood-forming and blood-destroying mechanisms are reacting to the thyroid feeding.

CHANGES IN THE RED-BLOOD-CELL PICTURE

In the case of the red blood cells, the thyroid feeding seems to cause (1) a drop in the total count followed by a rise with slight fluctuations which waver about or rise somewhat above the normal level, and (2) an initial rise in the reticulated cells followed by a fall, a rise, and a fall, though almost without exception the percentage of reticulations constantly lies above normal. The general correlation of these facts may be gathered from smoothed curves made from averaged data and the "tendencies" indicated (Fig. 18). It is difficult to compare this picture with the findings of previous investigators because of differences in procedure, animals used, and dura-

tion of the study. Consecutive daily blood counts after thyroid feeding in animals are rare, one of the few found being the paper of Lim, Sarkar, and Graham Brown (1922) on rabbits. Even here neither curves nor data showing variation in red cells in blood counts or in percentage of reticulated cells are given, but the general statements are made (pp. 234, 235, 237) that in young animals (weight, 500–600 grams, 5 days of thyroid feeding) "a doubtful increase of erythrocytes" is evident, while older ones (weight, 1,000–1,500 grams, 16 days of thyroid feeding) "showed little change of the erythrocyte count," and "the total blood counts show no definite change with regard to the red blood corpuscles" in still older animals (weight, 2,000–2,500 grams, 8–12 days of thyroid feeding). These data appear to support those of previous investigators (on dogs, rabbits, cats, guinea pigs, and man) whose work they cite, although rarely a polycythemic condition is mentioned. Nevertheless, in the rats fed thyroid in the present experiment before the normal condition or slight polycythemia occurred, an anemia appeared.

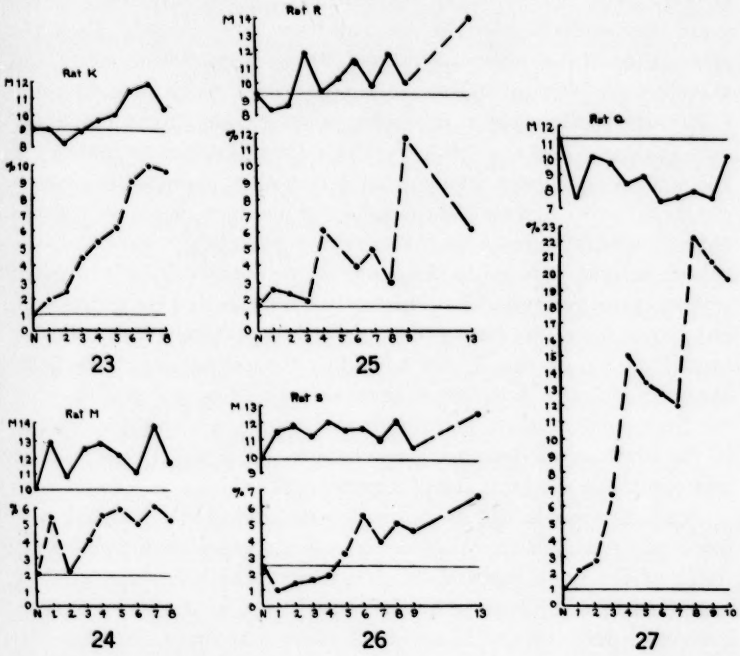
Such an anemic condition is in line with the findings of Zondek and Koehler (1926), who gave small doses of thyroid to healthy individuals and in some cases secured a marked anemia which lasted 10–12 days. They also observed a similar reaction in three other persons (ill individuals) after like treatment—in one case the anemia appeared after 3 days, in another after a month—and they make the assumption that the thyroid had stimulated the spleen to hold the red cells and thus to keep them out of the peripheral circulation. While this reservoir function may be heightened in the thyroid-fed rats, it seems more likely that increased red-cell destruction is the potent factor in lowering the total red count. Although neither phagocytosis of red cells (Rowley, 1908; Rous, 1923) nor fragmentation (Rous, 1923) were observed in the circulating blood of the thyroid-fed animals, it is hardly legitimate to exclude at least the latter of these possibilities. However, the fact that red-cell destruction goes on normally and in certain diseased conditions in increased amount in the spleen (Rous, 1923; Krumbhaar, 1926) makes heightened activity of this organ seem likely. Moreover, in rats and guinea pigs, in addition to red-cell destruction in the spleen, certain cells in the bone marrow and exceptionally in lymph nodes may ingest

erythrocytes under normal conditions; and it is probable that the liver may also be a seat of red-blood-cell destruction judged from the bile pigment found in the Kupffer cells (Rous and Robertson, 1917). These findings in normal animals are corroborated by experimental and clinical evidence. Speidel (1926*b*) describes an increased destruction of erythrocytes with large numbers of erythrocytic fragments in the spleen, a rise in the number of mononuclear phagocytes, an unusually large amount of erythrocytic débris, and melaninlike pigment in the liver cells of amphibian larvae which are undergoing forced metamorphosis due to thyroid feeding. In plethoric animals and those with simple anemia, greatly increased fragmentation of red cells takes place, as evidenced by examination of the spleen (Robertson and Rous, 1917). Peabody and Brown (1925) claim that phagocytosis of red cells may occur in normal bone marrow to a very limited extent and that an abnormal amount often takes place in hyperplastic bone marrow in some cases of pernicious anemia and other diseased conditions; while Doan (1926) demonstrates by the supravital method that the increased phagocytosis in the bone marrow in pernicious anemia is usually carried on by endothelial cells, although he occasionally has observed polymorphonuclear leucocytes containing red blood cells in the bone marrow.

In order to find out whether the spleen was a causative factor in the lowered red blood count immediately following thyroid feeding, four animals were splenectomized,[1] the operations being performed with no loss of blood. Beginning 1 day after the operation in two cases (rats K and M) and 8 days afterward in the other two (rats R and S), the animals were fed 4 grains of thyroid daily, and blood counts (a total red-cell count, a reticulated red-cell count, and a haemoglobin determination) covering a period of 8 or 9 consecutive days were made. The difference in time elapsing between the operation and the thyroid feeding did not seem to affect the results. While normally anemia follows splenectomy in mammals, perhaps "caused by a loss with the spleen of some substance which exerts a stimulating influence on the bone marrow" (Krumbhaar, 1926, p. 171), the data in the splenectomized thyroid-fed animals indicate that an ir-

[1] The authors wish to thank Dr. Christianna Smith for performing the splenectomy operations.

regular polycythemia accompanied by a moderate increase in the percentage of reticulated red cells occurs in every case (Figs. 23–26). (In two of the rats a slight but transient drop in total count appeared within a day or two after thyroid feeding [Figs. 23, 25, rats K and



FIGS. 23–27.—Curves showing total red count ————, and percentage of reticulated cells — — —, in rats K, M, R, S, respectively, which were splenectomized and fed 4 grains of thyroid daily and of rat Q fed 4 grains of thyroid daily but not splenectomized. Days on abscissa, millions (M) of red cells per cu. mm. and percentage of reticulated cells on ordinate. Lines paralled to abscissa indicate levels of normal count taken the day preceding thyroid feeding.

R]; and in one, rat S, the reticulated cells were below normal for a few days.) The red count was still above normal at the thirteenth day, when the last determinations were made, in rats R and S being respectively 4.7 and 2.7 millions above normal. It seems evident that this increase in red cells above normal in splenectomized thy-

roid-fed rats in contrast to the decrease in non-splenectomized thyroid-fed ones (Fig. 27; also Figs. 15, 16, 17) is due in the main to the lack of the spleen, the principal organ for erythrocyte destruction. Thus the assumption appears warranted that at first the spleen is stimulated by the thyroid to increased destruction of the red cells beyond the production in the bone marrow and probably when the stimulation of the bone marrow (see below) is just taking effect and therefore the lowering of total red count occurs. Such an assumption is further supported by Lim, Sarkar, and Graham Brown (1922, pp. 240, 241), who find that "the endothelial reaction is best marked in the spleen, and phagocytes loaded with yellow pigment (haematoidin?) are more often seen in the spleen of old animals fed on thyroid than in normal animals of the same age (weight)." However, the splenic activity may not be the only factor in the initial decrease of erythrocytes in thyroid-fed animals, as is suggested by the drop in red blood count noticed in two of the splenectomized thyroid-fed rats (Figs. 23, 25, rats K and R) and by the irregularity in the daily counts of red cells in all four of these animals (Figs. 23–26). Possibly the liver, bone marrow, and lymph nodes are functioning somewhat in the erythrocyte-destroying mechanism (cf. Speidel, 1926*b*), but this point has not been tested experimentally.

The increase in reticulations accompanying the lowered red-blood-cell count in thyroid-fed animals indicates an increased activity of the bone marrow. Such activity is well demonstrated by Lim, Sarkar, and Graham Brown (1922), who in sections and marrow counts find certain changes in the bone marrow of their thyroid-fed rabbits, such as absence of fat, congestion, increase in numbers of cells, and numerous mitotic figures (these agree with previous findings on bone marrow of dogs, cats, rabbits, and guinea pigs fed thyroid cited by Lim, Sarkar, and Graham Brown). In general, polymorphs are always increased, most noticeably in young animals, while small mononuclears tend at first to decrease and then to increase. There is a particularly close correlation of the polymorphs in marrow and blood counts. The small mononuclears are thought by these investigators to be the forerunners of erythrocytes and thus to have much to do with the maintenance of normal numbers of red cells in the blood. They do not mention reticulations in red cells in

the circulating blood, but this condition is admittedly recognized as indicating immature forms of erythrocytes.

How the thyroid feeding brings about increased stimulation of the bone marrow is uncertain, but three possible suggestions occur. (1) The heightened rate of metabolism which is characteristic of thyroid-fed animals with its resulting rapid internal respiration requires a more efficient transportation of oxygen and therefore an increased number of red blood cells, and the bone marrow probably responds to this demand. (2) It may be stimulated to erythrocytic production by increased carbon-dioxide content of the blood. In analyzing the effects of feeding thyroid to frog larvae, Jordan and Speidel (1924*a*, p. 401) state that the thyroid causes an increased metabolic rate which "leads to increased formation of acid metabolites, including especially carbon dioxide" and that "the increased concentration of carbon dioxide acts as a hormone to stimulate more rapid respiratory rate and more rapid erythrocytic differentiation." This point of view is in line with that of several workers (see Sabin, 1928, p. 201) and especially of Dallwig, Kolls, and Loevenhart (1915), who have found that the decrease in oxygen tension of respired air and also the increase of carbon-dioxide tension will stimulate the bone marrow, causing a marked rise in erythrocyte production within 5–7 days in rats, dogs, and rabbits. (3) The destruction of the red cells in the spleen and perhaps to some extent in the bone marrow, lymph nodes, and liver (and by fragmentation?) causes a still greater demand for erythrocytes. It is interesting to note here Eddy's suggestion (1921) that the splenic cells may elaborate the disintegrating erythrocytes which they have removed from the circulation into an internal secretion which may reduce the resistance of red blood cells (therefore possibly aiding in the initial lowering of the blood count), and after being modified by the liver may stimulate the erythrogenic activity of the bone marrow.

With the thyroid feeding initiating these three factors to stimulate the bone marrow, the rate of output of erythrocytes is evidently more rapid than the rate of differentiation, and immature cells (reticulated and some nucleated) are put forth into the circulating blood. However, in spite of the increased amount of production judged by the percentage of reticulations, the total number of eryth-

rocytes in the peripheral blood is at first decreased, and the lowering of the total count must be due to a destruction greater than the production of these cells. This initial excess destruction is shortly overcome, however, because reticulations continue to rise and the total number of red cells starts to increase as well (Fig. 18).

The steady rise in total count soon becomes associated with a drop in the number of reticulated cells. This is somewhat confusing, since the drop in percentage of reticulations implies (1) that these cells are being destroyed at a more rapid rate than they are being produced or (2) that they may be maturing into the adult form in the circulating blood or (3) that they are being put out into the circulation by the bone marrow at a decreased rate while the rise in total count suggests a still-stimulated bone marrow. Although there is no evidence that either a raising or a lowering of the activity of the cell-destroying mechanism after its first reaction has occurred, the increase in total count inclines one to the opinion that it may have, and such a period of lessened activity after the initial stimulation to cell destruction would be in accord with the recovery usual after excessive activity in many organs. Therefore the first possibility does not seem to explain the drop in percentage of reticulated cells.

The possibility that the reticulated cells are maturing into the adult form in the circulating blood is supported by the fact that the majority of the reticulations present are of the rather faint type characteristic of more mature stages (Plate I, Figs. 6, 7). Although it is not definitely known that maturation of reticulated cells occurs in the circulating blood, it is suggestive that Pepper (1922), in a vital study of human and rabbit reticulated cells, finds that the reticulations persist for a period of 18–72 hours in defibrinated blood kept at body temperature. He is of the opinion that this may give a hint as to the duration of reticulations in normal blood. In these experiments the somewhat longer time which it takes for the reticulations to disappear (their decrease covering several days) may be due to the great amount of basophilic substance within the cell and perhaps to the variation in the blood of different mammalian species. Thus the second suggestion still remains as a possibility in the reduction of the reticulated cells.

In spite of the fact that the total red count is increasing and the reticulations remain decidedly above the normal, which implies a still-stimulated bone marrow, the indications are that the activity of that organ has dropped from its highest point (though still above normal) both because the number of reticulated cells is lower and because the reticulations represent the more mature stages and because an interruption of the rise occurs in the drop at Point 3 (Fig. 18). The condition of the reticulated cells, although the possibility of a simultaneous maturing in the circulating blood is not eliminated, seems to hint that the bone marrow has adjusted itself somewhat to the stimuli (perhaps again an expression of fundamental rhythm, recovery after excessive activity), and while still producing numbers of cells in excess of the normal, is turning them out in a more differentiated stage. Also the persistent and rising polycythemia caused by thyroid feeding in splenectomized rats indicates a continued stimulation above normal by the thyroid, although in such animals the polycythemia is in part due to the lack of the spleen.

Another method of increasing the red-cell number besides a decrease in splenic destruction and the stimulation of bone marrow may be the transformation of lymphocytes into erythrocytes. Although the conception of the origin of red cells and white cells from separate stems (Cunningham, Sabin, and Doan, 1925; Sabin, 1928) is accepted by many investigators, others are definitely of the opinion that lymphocytes may differentiate into red blood cells. Jordan and Speidel (1923*b*, 1924*a*, 1924*b*, 1925) believe that the erythrocytes of the anuran and urodele Amphibia arise from large lymphocytes in the spleen. They also (1923*a*) state that in mammals lymphocytes from lymph nodes may find their way into the bone marrow where they serve as blood mother-cells, giving rise especially to erythrocytes; and more recently Jordan (1926) has described five mammalian lymph nodes from dog and man in which lymphocytes were transforming into erythrocytes. Badertscher (1915, p. 484), from his study of the development of the thymus in the pig, finds that "the free red blood cells and eosinophile cells found in both interlobular septa and the thymic lobules are derived from lymphocytes *in situ*," although he is not sure that any of such erythrocytes enter

the circulating blood. Likewise, Latta (1921)[1] and Maximow (1924,[2] 1927) have stated that under certain conditions lymphocytes may act as the ancestors of erythrocytes; and Lim *et al.* (1922) have discovered in the bone marrow of their thyroid-fed rabbits increased numbers of small mononuclears (resembling lymphocytes), which they believe give rise to red blood cells and not to lymphocytes. Bloom (1926) also upholds the potentiality of the lymphocytes and records his belief that in the guinea pig lymphocytes are cells in a temporarily inactive state with unrestricted capacity for development. After passing through the blood stream they acquire the ability to develop, and small lymphocytes become large lymphocytes, and these in turn give rise to myelocytes, megocaryocytes, and erythroblasts.

The existence of a high percentage of lymphocytes during a low erythrocyte count and the decrease in their number correlated with the increase in red blood cells in these studies (Fig. 18) are strongly suggestive of the possibility of deriving erythrocytes from lymphocytes. With this reaction Speidel's report (1926*a*) on the thymus of thyroid-treated tadpoles would agree, since he observed a lymphoid reaction of the thymus to thyroid treatment; and he believes that these lymphocytes function as mother-cells of erythrocytes during the critical period of high metabolism. In the present study also, it was found that the lymph nodes in the neck and axillae of three rats dying after 10 days of thyroid feeding had the gross appearance

[1] Latta (1921) from his study of the rabbit considers that environmental conditions are probably at the root of the differentiation of lymphoid haemoblasts into erythroblastic or granuloblastic cells. "If the association between the lymphoid hemoblasts and the blood vessels is very close and the conditions necessary for the transudation of materials from the blood stream (plasma) are very good, erythropoiesis occurs. If the association is not so close or the transudation conditions present to a lesser degree, granulopoiesis results" (p. 200).

[2] Maximow (1924, p. 545) states: "In abnormal conditions—after heavy blood losses, in blood diseases—the homoplastic process in the bone marrow may become unsufficient; the hematopoiesis is pushed backward and the heteroplastic formation of myeloid elements my be called forth—the young forms, the erythroblasts and myelocytes are not only proliferating and differentiating to mature forms, but they originate anew, as in the embryo, from primitive, indifferent cells, with a large clear nucleus and a basophilic, homogenous protoplasm, which histologically are identical with the larger types of lymphocytes in the lymphoid tissue."

of haemolymph nodes,[1] which seems to agree with Jordan's observation (1926). From this line of reasoning, the steady increase in red blood cells at a time when the evidence from blood counts indicates that the bone marrow is not maintaining its highest peak of activity though still above normal, and the simultaneous decrease in lymphocytes, may be due in part to the derivation of erythrocytes from lymphocytes.

Thus from the foregoing analysis the return to normal or the slight polycythemia which is evident in these thyroid-fed rats is due probably (1) to an overstimulated bone marrow, evidenced again by a second period of reticulated cell formation which does not reach the heights of the first (Figs. 15, 16, 17), and possibly (2) to a lowering or merely normal red-cell destruction on the part of the spleen in the presence of increased production. (There is of course the possibility that the spleen is reverting to its fetal function and contributing to the red-cell number [Sabin, 1928].) It seems likely that the same factors suggested earlier (p. 207) are still at the root of the increased erythrocyte production by the bone marrow, since there has been no cessation in feeding thyroid. There is no evidence for a lowered splenic activity in red-cell destruction. In fact, if the first reaction of the spleen to the thyroid is increased destruction, it seems likely that such activity would continue at least at a level somewhat above normal rather than drop below normal, and of course this could happen and a normal or increased red-cell number be present if the production of red cells was sufficiently in excess of the normal. Moreover, the data from thyroid feeding experiments in amphibians (Speidel, 1926*b*) and in mammals (Hoskins, 1916; Cameron and Carmichael, 1921*a*, 1921*b*) indicate a hyperplasia of the spleen; and Lim *et al.* (1922), as earlier stated (p. 206), find "phagocytes loaded with yellow pigment (haematoidin?)" in some of their older thyroid-fed rabbits. However, histological examination of this organ in certain thyroid-fed rats (Cameron and Carmichael, 1921*a*,

[1] These nodes have not yet been examined histologically, but it is interesting to note that MacMillan (1928, p. 167) finds that "red lymphatic nodes . . . . are inconstant variable structures in the rat and guinea-pig" and that she did not observe any haemopoiesis in such nodes although certain "groups of plasma cells sometimes superficially resembled its early stages."

1921*b*) revealed no difference from the normal controls. (3) The possibility of the transformation of lymphocytes into erythrocytes may also be aiding in the normal or somewhat supernormal red count, since the lymphocyte numbers seem to lie rather below the normal in spite of the supposed hyperplasia of lymphatic tissue after thyroid feeding.

It has been noticed that the haemoglobin content of the blood throughout the increased erythrocyte count has not risen in conformity with the number of erythrocytes, the ratio of haemoglobin to red cells being slightly lower than normal. It is evident both in vital and fixed smears that this lowering is due to an insufficient amount of haemoglobin in the reticulated cells. In general, it may be said that the amount of reticulation and the haemoglobin content of the reticulated cells are inversely proportional.

CHANGES IN THE WHITE-BLOOD-CELL PICTURE

It will be recalled that the reaction of the white blood cells to the thyroid feeding consists of a series of rises and falls which have the general correlation with the red-cell count of a high white count with a low red count, and vice versa (Fig. 18). The first increase in total count (Point I) is largely due to a rise in leucocytes (Figs. 18, 19, 20, 21) and appears to be similar to the increase in polymorphs in blood counts of rabbits after 5–8 days of thyroid feeding (Lim *et al.*, 1922). Such an increase must result from the stimulation of the leucogenic centers of the bone marrow by the thyroid feeding, as is clear from the increased numbers of polymorphs in marrow counts (Lim *et al.*, 1922), "shift to the left" in the Arneth count in rabbits (Ponder, 1924, 1926), and the finding of myelocytes, the ancestors of neutrophiles, and many "ring nucleus" neutrophiles—possibly types younger than Type I, Arneth count—with large numbers of mitochondria in these thyroid-fed rats. It coincides with the beginning of the rise in percentage of reticulated cells from the stimulated erythrogenic centers. The lymphocytes are on the increase also and are largely responsible for the second high point (Point III), which is separated from Point I by a small drop. The higher lymphocyte numbers are probably due to the beginning of the stimulation of the lymphoid tissue by the thyroid treatment, which charac-

teristically ends in lymphoid hyperplasia (Speidel, 1926*b*, in tadpoles; Hoskins, 1916, in rats; Cameron and Carmichael, 1921*a*, 1921*b*, in rats and rabbits; MacCallum, 1918, in cases of exophthalmic goiter in man).

The leucocytic and lymphocytic drops which appear in Point IV, together with a drop in the total white count (Fig. 18), are not easy to understand. In both cases they may be "recovery periods" in the bone marrow, or they may indicate some form of loss of blood or redistribution of cells. No fragile cells were observed in the circulating blood at this time, and Bunting and Huston (1921) doubt whether lymphocytes disintegrate in the circulation in significant numbers. Neither does phagocytosis or destruction of leucocytes seem normally to be a function of any of the organs. However, Carrel and Ebeling (1926) have seen polymorphonuclear neutrophiles ingested by macrophages in tissue cultures. The possibility of redistribution seems more plausible, especially for the leucocytes; and they may have been carried to some part of the body where there is a functional demand for them and thus appear in lessened numbers in the peripheral circulation. Broun (1922), in dogs, and Isaacs and Gordon (1924), in man, have shown that a redistribution of red and white cells takes place during exercise, although the latter find an increase (not a decrease) in the number of polymorphonucleated leucocytes in the peripheral circulation. In the increased metabolism of thyroid feeding, however, the demands of the internal organs (many of them—such as heart, liver, spleen, adrenals, and kidneys—hypertrophy, according to investigators [Hoskins, 1916; Cameron Carmichael, 1921*a*, 1921*b;* and others]) may call the cells from the periphery. Bertelli and Falta (Baker, 1924) also have found an excess of neutrophilic cells in the blood of the liver in dogs fed thyroid, and Jordan and Speidel (1923*b*) point out that there is a variation in leucocytic numbers, particularly in regions of progressive and regressive changes, in thyroid-fed tadpoles with their increased metabolism. An examination of the liver and digestive tract in thyroid-fed rats might throw some light on this point, although it is interesting that histological examination of hyperplastic tissues such as liver, kidney, heart, spleen, and adrenals of thyroid-fed rats (Cameron and Carmichael, 1921*a*) and of spleen and hypertrophied lymphatic

tissue from the glands of the neck of thyroxin-fed rats (Cameron and Carmichael, 1921*b*) revealed no differences from such tissues in normal control animals. Neither do the histological findings of Lim *et al.* (1922) on lymphatic organs throw light on this subject.

The third high total count (Point V, Figs. 18, 19, 20, 21) which is largely caused by an increase in lymphocytes, although the leucocytic count is advancing also, represents still heightened activity on the part of the bone marrow (with the continued presence of myelocytes and increasing numbers of "ring nucleus" neutrophiles with mitochondria in the blood) and of lymph glands after the "recovery period." After Point V, which falls within the counts made on 10 consecutive days, there is a steady drop in leucocytes and lymphocytes, which seems to reach a low point (below normal) in the total count some days later (Point VI). This also may be another "recovery period"—the smoothed curve in Figure 18 is strongly suggestive of such an interpretation.

In continued counts on certain animals, however, a subsequent rise in white count occurs which lodges the total within or slightly above the normal limits—occasionally a count seems to be dropping—while the leucocytes increase and lymphocytes decrease. These tendencies may be illustrated by reference to Table III, in which counts on the three rats (E, F, G, fed 2 grains of thyroid daily) were continued longest. These show a variation in the behavior of the total count—E is rising, F and G falling, although F is still above normal—but the leucocytes are on the increase and lymphocytes on the decrease in all three of the animals. Heightened activity with insufficient time for differentiation must still be occurring in the bone marrow, judged again by myelocytes and "ring nucleus" neutrophiles with mitochondria in the blood, to account for the rise in leucocytes; but the dropping in lymphocytes seems inexplicable if lymphoid hyperplasia, which ought to be indicative of an increase of numbers of lymphocytes, has occurred, as is usual in thyroid-fed animals. Perhaps a part of the lymphocytes are being transformed into erythrocytes as earlier suggested, and perhaps they are being destroyed in such excessive numbers that even increased production cannot maintain them at or above the normal level. The likelihood that they may be eliminated by way of the gastrointes-

tinal tract (Bunting and Huston, 1921) seems plausible, especially when the digestive disturbances so often associated with thyroid feeding (Carlson, Rooks, and McKie, 1912; and others) and which were seen to some extent in rats used in these experiments are considered.

These findings agree only in part with the references recorded in the introduction to this paper, with those cited by Lim *et al.* (1922) or by Whitney (1928), where lymphocytosis is said to be a characteristic reaction to thyroid feeding or in hyperthyroid states (exophthalmic goiter) and red and white counts show relatively no

TABLE III

BLOOD COUNTS IN RATS FED 2 GRAINS OF THYROID DAILY

Rat	Days of Thyroid Feeding	Red Count Total	Percentage of Reticulated Red Cells	White Count Total	Leucocytes	Lymphocytes
E	34	+1,300,000	+2.8	Normal	+1,600	−1,300
	48	+1,500,000	+6.4	+2,000	+7,100	−4,000
F	34	+1,200,000	+6.0	+3,900	+5,600	−1,300
	48	+3,100,000	+3.6	+1,000	+6,900	−6,000
G	44	+2,500,000	+0.8	− 200	+ 700	−2,200
	48	+2,000,000	+3.4	−3,200	+2,100	−5,900

+ Indicates count above, − below, normal.

change. (Occasionally polycythemia and leukopenia are recorded.) Here, after an anemic period with accompanying lymphocytosis and leucocytosis, the red count returns to normal or above, while a normal (or slightly higher or lower) total white count appears to be present in which leucocytes lie above the normal and lymphocytes below. It is true that the number of animals is too small to present conclusive evidence on the lymphopenia, but nevertheless the reaction was consistent in the three rats (E, F, G) at 53 days and indicated in others whose counts were not continued so long. The condition may, however, be due to some other cause and not to the thyroid feeding. It is interesting to note that Zondek and Koehler (1926) report a case of thyroid administration in exophthalmic goiter which was followed by a decrease in lymphocytes, and Naegeli (1923, p. 139)[1]

[1] "Lange durchgeführte Untersuchungen in meinem Institut durch Hanhardt ergaben bei Parenchymatösen Strumen des Menschen und des Hundes keine konstante Lymphozytose, auch nicht auf Thyreoidin (oral oder subkutan) oder Jod."

does not appear to believe that lymphocytoses are the invariable accompaniments of hyperthyroidism.

There is an additional factor which should be mentioned in considering the effect of thyroid feeding on the blood picture, although no data are available on this point. Throughout the attempt to analyze the variation in blood-counts, only the reaction of the blood-forming or blood-destroying mechanism to the thyroid feeding has been considered. This may, however, be only a part of the story, and the thyroid may be acting on some other endocrine gland or glands, and it or they may in turn be playing some rôle in the changes in the blood picture.

SUMMARY

1. The blood of normal and thyroid-fed albino rats from two stocks (Wistar Institute and Mount Holyoke) was studied by making (1) total counts of red and white cells, (2) determination of percentage of reticulated red cells, (3) differential counts in supravitally stained films (Sabin's method) and in fixed smears (Wright's stain), and (4) haemoglobin determinations over periods including consecutive days. The average total red-cell count of the Wistar stock was: ♂, 9,921,661; ♀, 9,262,314; Mount Holyoke stock, ♂, 9,530,000, ♀, 9,070,000. The average total white count for both sexes in the Wistar stock was 12,959.5 cells, and in the Mount Holyoke stock, 10,373.5.

2. A type of cell called the "unknown cell," which did not agree with any previous descriptions, was found in the normal rat blood. Because of the similarities in appearance and behavior of this cell to the small lymphocyte, it was included with them in normal and experimental data. The percentage of lymphocytes, including "unknown cells", was about twice that of leucocytes.

3. Following experimentally produced hyperthyroidism in rats, there is a haemopoietic reaction which within varying times follows a definite pattern, as judged by blood counts on consecutive days or at 4- or 5-day intervals. There is a drop in the total number of red blood cells and an increase in percentage of reticulated cells after thyroid feeding, which is finally followed by a normal or somewhat higher red count in which the percentage of reticulations is above normal. The white-blood-cell picture consists of a series of

rises and falls which have a general correlation of a high total white with a low total red count, and a high red with a low or normal white count. The data at the end of the experiment indicate a normal (or slightly higher or lower) total white count, with a differential picture of leucocytosis and lymphopenia.

4. From an analysis of the blood counts it appears that thyroid feeding stimulates the bone marrow to increase production of blood cells, both erythrocytes and leucocytes, and to bring about increased cell destruction in the spleen during the first part of the feeding with *possibly* a later inhibition of splenic activity. From other investigations it seems plausible that it effects a hyperplasia of the lymphatic tissue and lymphocytosis is to be expected. In these experiments the findings indicate that the lymphocytosis appears only at first and that a lymphopenic condition exists after continued thyroid feeding. The suggestion is made that this may be due to the formation of erythrocytes from lymphocytes, thus contributing to the red-cell number, although an excessive lymphocytic destruction in spite of increased production cannot be eliminated as a possibility.

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THE RELATION OF DENSITY OF POPULATION TO RATE OF REPRODUCTION IN PARAMECIUM CAUDATUM[1]

(One figure)

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INTRODUCTION

THE relation of density of population to survival and rate of reproduction in protozoans has attracted much attention recently. Woodruff in 1911 found in working with *Paramecium* that increased density decreases the reproductive rate. He found that the rate of reproduction of *Paramecium aurelia* and *P. caudatum* is influenced by the volume of the culture medium and that the greater the volume the more rapid is the rate of division. "Paramecia excrete substances which are toxic to themselves, and these substances are more effective when organisms are confined in limited volumes of culture fluid" (1911, p. 581).

Jennings, in work on the effects of conjugation in *P. aurelia*, found indications of lowered vitality in slide cultures. "I believe that everyone who has had extensive experience with experiments of this sort will agree with me that long continued cultivation on slides does produce a depressed condition. There are some stocks that will not stand it at all, though they live perfectly in mass culture" (1913, p. 325). Myers (1927, p. 38) presents evidence to show that a single *P. caudatum* requires from 0.2 to 0.4 cc. of flourishing hay infusion in order to unfold its full potentialities, and suggests that the lack of sufficient medium is probably the cause for their failure to thrive in slide cultures.

In 1921 and 1922, T. B. Robertson described opposed results with the Australian ciliate *Enchelys farcimen* and with another ciliate which he called *Colpidium colpoda* and which he later had identified as *Colpoda cucullus*.

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> When two infusoria (Enchelys or Colpidium) are isolated together into the same volume of culture medium, the numbers of individuals produced within a given period is not double but much more than double the number of individuals which is produced in the same time in a culture of equal volume which was originally inoculated with a single individual.....
>
> Evidently some substance issues from the cells which has the effect of accelerating multiplication. When more than one individual is initially present the concentration of this substance is higher and the rate of multiplication is, in consequence, enhanced [1924, p. 108].

This he called the "allelocatalytic" effect. At first he thought that old culture fluid contained no substances that were toxic to the infusoria isolated into it from young cultures; but as evidence accumulated, he decided that "the ultimate cessation of reproduction in old cultures is attributable also to the accumulation of a product of growth, and possibly the same product that was originally responsible for the acceleration" (1924, p. 108).

The allelocatalytic effect described by Robertson in *Enchelys* and *Colpidium* caused other investigators to make studies under similar conditions using various other organisms. Cutler and Crump (1923) tested for allelocatalysis in *Colpidium colpoda*. One, two, three, and four animals were isolated into drops of various sizes from 0.5 to 9.0 cu. mm. Their results indicate that animals in larger drops with a low initial concentration reproduce more rapidly than those in the smaller drops and that there is no allelocatalytic effect produced by the second, third, or fourth animal in the drop.

Similarly, G. L. Peskett (1924), in working with sterile cultures of yeast, finds that the growth of the cultures initially containing one, two, or three cells is in proportion to the number of living cells present but is not accelerated by the presence of another yeast cell.

Greenleaf, working with *Paramecium caudatum*, *P. aurelia*, and *Pleurotricha lanceolata*, in 2–40 drops, concludes that "the data in both sets of experiments are entirely concordant and afford not only no evidence that the cells produce a substance which accelerates the division rate, but also supply further evidence that the excretion products of cells are inimical to reproduction" (1924, p. 406). In a later report (1926) he says, "Thus all the experiments described point unequivocally to the conclusion that all three species of infusorians tested divide faster, within certain limits in larger volumes

of culture medium" (p. 153). In his allelocatalysis experiments, one and two *Stylonychia pustulata* were inoculated into both 2 and 5 drops of medium. He found that one-animal controls usually divided faster in both the 2- and 5-drop cultures than did the experimental animals which were bred together. There was no allelocatalytic effect, and there was indication that "in the larger volume the depressing effect of the presence of the two animals is less marked than in the smaller volume" (1926, p. 154).

Robertson criticizes the work of Cutler and Crump and Greenleaf because unwashed animals were used in the experiments, and states that "allelocatalytic effect is frequently absent or replaced by actual retardation in unwashed cultures, while it becomes progressively more manifest the more thoroughly the cultures are washed" (1924, p. 152).

Cutler and Crump later report the results of experiments on washed *Colpidium*. "In *Colpidium colpoda* allelocatalysis has not been observed in cultures either from washed or unwashed individuals" (1925, p. 453). Peskett also repeated his experiments with yeast and found that with thorough washing of the cells he still did not obtain the allelocatalytic effect (1925).

Early in his work Robertson raised the question whether the observed allelocatalysis was due to the infusoria or to the associated bacteria. It was found that bacterized infusions also showed the allelocatalytic effect.

> Cutler and Crump, however, have shown that the growth of Colpidium is much more facilitated by the "contaminating bacillus" which was found by them to be associated with this organism than it is by Sarcina which they employed as a food organism. This suggests the possibility that Colpidium and other ciliates can live only upon particular species of bacteria, and that they modify the nutrients available for the food species of bacteria. . . . .
>
> Whether the allelocatalytic effect originates with the ciliates, or with associated food organisms, however, it remains a mutually accelerative effect of contiguous organisms upon their reproductive rates [1927, pp. 4, 5].

Calkins (1926, p. 206) records the results of 60-day experiments with *Uroleptus mobilis* in which one, two, three, and four individuals were inoculated into 1 drop of fresh medium. The division rate was found to be inversely proportional to the initial number of individuals inoculated.

Myers (1927) worked with washed *Paramecium caudatum* and found that "increasing the number of individuals present in a given volume does not increase the rate of reproduction either from the beginning of the cultures or after the first fission. On the contrary, it decreases it markedly" (p. 14). He found, as did Cutler and Crump and Peskett, that washing the individuals before introducing them into the new culture medium makes practically no difference in the results. "The lack of allelocatalytic effect in unwashed individuals is not due to the fact that they were not washed, for those thoroughly washed show it equally" (p. 15). Populations of cultures were counted at intervals of 6, 6, and 12 hours. "In all cases the greater the number of individuals per volume, the less the reproductive power" (p. 42). A more detailed discussion of this part of Myers' work will be given later.

Yocum (1927) reported allelocatalysis in work on *Oxytricha*. He isolated unwashed animals to 4 and 10 drops (100 drops to 1 cc.) of sterile beef medium and counted the animals after 24 hours, finding that in all cases the division rate in 4 drops exceeded that in 10 drops by more than 10 per cent.

MATERIAL AND METHODS

Paramecium caudatum was used in all my experiments. Laboratory wheat cultures furnished the animals used in the work. For stock cultures, 25 cc. of bacterized hay medium was inoculated with several drops of the preceding culture. This was done at 2- or 3-day intervals. Beef, wheat, and hay medium were tried, but hay infusions were found to be the most constant and favorable for the experiments. These were prepared by boiling 5 grams of Timothy hay in a liter of well water, from the Whitman Laboratory well. When cool, the liquid was filtered to remove small particles of hay. It was then put into 25 and 50 cc. bottles. The uncorked bottles and corks were kept in the boiling water of a Castle sterilizer for 2 hours. The bottles were corked when hot, and used as needed. Twenty-four hours before starting an experiment a bottle was opened and the hay medium was seeded with a drop ($\frac{1}{24}$ cc.) of the bacterized medium of the preceding day and allowed to stand open.

In the earlier experiments with small volumes, the animals were

isolated to depression slides. In the experiments using larger volumes, up to 40 drops, animals were kept in salt cellars. Evaporation was avoided effectively by keeping the slides and salt cellars on wet sand in covered crystallization dishes. Experimental and control animals were placed in twin depressions on the same slide; or when salt cellars were used, they were kept in the same crystallization dish.

Animals were isolated by means of a drawn-out pipette; they were washed by re-isolation into fresh medium (5 drops). Throughout the experiments 24 drops were equal to 1 cc.

EXPERIMENTAL RESULTS

TEMPERATURE-AXIAL RELATIONS TO RATE OF DIVISION

The usual method of comparing individuals of the same clone was not followed in this work, because early in the course of the experiments it was found that there was a marked difference in division rate of animals from the same clone. In an attempt to suppress this difference, the rate of sister-cells was compared. A difference was still apparent.

The next step was to isolate the two daughter-cells of a large number of animals and at the following division re-isolate the fission pieces of two sets with approximately equal rate of division and time of division for experiment and control. In the isolation of the two daughter-cells, records were kept concerning whether they had been anterior or posterior pieces of the parent-cell. An interesting fact became apparent. In those cells isolated in the evening, the posterior piece usually divided first; while in those isolated in the morning, the anterior piece usually divided first. This suggested a possible temperature-polarity-division relation, which was investigated at some length since it is imperative in experiments to test allelocatalysis to start with cells having a closely similar rate of division.

In 21 of the 24 cases isolated in the morning, the anterior piece divided first; in those isolated at night, in 11 of 16 the anterior piece divided last. To test this difference in division rate farther, the exact time of division of the fission products was recorded for three filial generations at three temperature ranges: in 15 cases animals were transferred from room temperature to 26°–30° C.; in 14 cases

they were held at room temperature, 18°–22° C.; and in 12 cases they were transferred from room temperature to 13°–17° C. Table I gives the detailed data for one set at each temperature range.

TABLE I

SHOWING TYPICAL EXAMPLES OF DIVISION TIME OF THE PROGENY OF ONE ANIMAL AT VARIOUS TEMPERATURES

1	2	3	4	5	6	7
Parent	Cell	Time	Cell	Time	Cell	Time
A. Low Temperature, 13°–17° C.						
2:20 P.M.	1	1:30 P.M. (23.10)	A	9:40 A.M. (20.10)	a	4:35 A.M. (18.55)
					b	4:30 A.M. (18.50)
			B	8:25 A.M (18.55)	c	3:06 A.M. (18.41)
					d	3:00 A.M. (18.35)
	2	11:45 P.M. (21.25)	C	7:00 A.M. (19.15)	e	1:38 A.M. (18.38)
					f	1:20 A.M. (18.20)
			D	6:45 A.M. (19.00)	g	1:15 A.M. (18.30)
					h	1:02 A.M. (18.17)
B. Temperature, 26°–30° C.						
11:24 P.M.	1	1:22 P.M. (13.58)	A	1:17 A.M. (11.55)	a	12:49 P.M. (11.32)
					b	2:18 P.M. (13.01)
			B	1:23 A.M. (12.01)	c	12:56 P.M. (11.33)
					d	1:21 P.M. (11.58)
	2	1:39 P.M. (14.15)	C	2:25 A.M. (12.46)	e	2:56 P.M. (12.31)
					f	2:59 P.M. (12.34)
			D	4:00 A.M. (14.21)	g	5:58 P.M. (13.58)
					h	6:15 P.M. (14.15)

TABLE I—*Continued*

1	2	3	4	5	6	7
Parent	Cell	Time	Cell	Time	Cell	Time
C. Normal Temperature, 18°–22° C.						
8:25 A.M.	1	8:29 P.M. (12.04)	A	10:30 A.M. (14.01)	a	8:58 P.M. (10.28)
					b	10:15 P.M. (11.45)
			B	11:06 A.M. (14.37)	c	10:00 P.M. (10.54)
					d	11:18 P.M. (12.12)
	2	9:04 P.M. (12.39)	C	10:40 A.M. (13.36)	e	10:48 P.M. (12.08)
					f	11:53 P.M. (13.13)
			D	3:30 P.M. (18.26)	g	4:22 A.M. (12.52)
					h	5:48 A.M. (14.18)

EXPLANATION OF TABLE I

Column 1. Time of division of parent-cell.

Column 2. Resulting cells (see Fig. 1).

Column 3. Time of division of cells of the first filial generation.

Columns 4 and 5. Same as columns 2 and 3, respectively, for the second generation.

Columns 6 and 7. The same for the third generation.

The numbers in parentheses give the length of time in hours between cell divisions.

Constant attention was required in the experiments for approximately 48 hours at the highest temperature and for over 72 hours at low temperature, in the effort to obtain the exact time of division of all progeny and to identify the fission products at the time of division and to re-isolate them to the fourth generation. Many other sets of animals were started but not finished because of the loss of one of the cells in transfer or because of the inability to follow and re-isolate the anterior and posterior cells with certainty on account of their rapid twisting at the time of division or their immobility after division. The dividing animals acted in two distinct ways: some spiraled rapidly, and others lay quietly at the bottom of the depression and at division gently separated and remained close to-

gether, often circling around each other. Both types of behavior made it extremely difficult to isolate anterior and posterior pieces with certainty.

Usually animals were examined about 8 hours after isolation and subsequently every hour until the first signs of division appeared. Approximately ½ hour before fission the animals broaden. After the broadening was noticed, they had to be watched constantly. The results of these temperature experiments have been summarized in a preliminary report (1927).

The order of division at high temperature is given first. The letters in tabular matter correspond to the letters in Figure 1. The numbers represent the order of division of the cells. In the division of the first filial generation at high temperature, the anterior piece always divided first. The division of the second filial generation occurred in the following order.

A	1	1	1	1	1	1	1	3	1	1	2	1	1	1	1	1
B	2	2	2	2	2	4	2	4	2	2	1	3	3	2	2	2	2
C	3	3	3	3	3	2	3	1	4	3	4	2	2	3	3	3	3
D	4	4	4	4	4	3	4	2	3	4	3	4	4	4	4	4	4

The "A" piece divided first in 88.8 per cent of the cases, and the "D" cell was last in 76.6 per cent.

As shown in the third generation, the "a" piece divided first in 80 per cent of the cases and in the other 20 per cent it came second; while the "e" piece, which is the anterior piece of the posterior cell produced in the second generation, divided first in 13.3 per cent of the cases. The "h" piece under these conditions divided last 93.3 per cent of the times.

a	1	1	1	1	1	2	1	1	1	1	1	2	2	1	1
b	3	3	4	5	2	6	4	2	4	5	5	4	3	3	4
c	2	2	3	3	3	1	3	3	2	2	3	3	6	2	2
d	5	5	5	6	7	3	7	4	6	6	6	7	7	4	3
e	4	4	2	2	6	4	2	6	3	3	2	1	1	5	5
f	7	7	7	4	8	5	5	7	7	7	4	5	4	7	6
g	6	6	6	7	4	7	6	5	5	4	7	6	5	6	7
h	8	8	8	8	5	8	8	8	8	8	8	8	8	8	8

There is no definite order evident at normal temperatures in the division of the third filial generation, but the recorded times of division give further evidence that all individuals from the same clone,

treated similarly, do not have the same rate of division in first, second, or third filial generations. It was found that two parents with approximately equal rates of division produce daughter-cells which

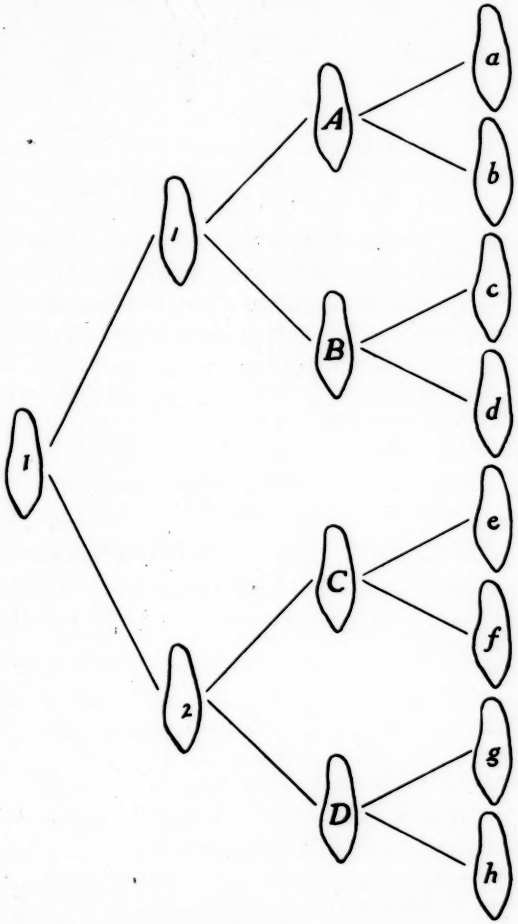

FIG. 1.—Showing cell lineage to third filial generation. Column 1 represents the parent cell; column 2, the first filial generation; column 3, the second filial generation; and column 4, the third filial generation.

also have similar rates. The third filial generation may show as much as 12 hours difference between the time of division of the fastest and slowest cells. The third filial generation in normal temperatures, divided as follows:

a	6	7	7	4	2	5	5	7	5	2	4	8	7	7
b	7	6	6	2	1	1	4	5	3	1	3	7	5	5
c	1	1	8	1	3	7	1	8	7	4	6	5	8	8
d	2	3	5	3	6	6	3	6	8	7	5	4	6	6
e	8	4	2	8	7	3	7	4	1	6	8	3	4	4
f	5	2	4	5	4	4	6	1	6	8	7	1	2	2
g	4	5	3	7	8	8	8	2	2	5	2	6	3	3
h	3	8	1	6	5	2	2	3	4	3	1	2	1	1

The order of division in lowered temperature is more definite, and for the twelve series follows. The first filial generation showed the fastest division in the posterior pieces consistently. In the second filial generation the "A" pieces were slowest 84 per cent of the time, and the "C" piece 16 per cent; while the "D" piece was the first to divide 75 per cent, and "B" 25 per cent, of the time.

A	4	4	2	4	4	4	4	4	2	4	4	4
B	1	3	1	3	3	2	3	3	1	3	3	2
C	3	2	4	2	2	3	2	2	4	2	2	3
D	2	1	3	1	1	1	1	1	3	1	1	1

In the third generation there is indicated a possibility of slight differential acclimatization in the low temperature. The order of division of the third filial generation in lowered temperature for the twelve series follows.

a	8	8	4	7	4	6	5	8	6	8	7	7
b	7	3	3	6	3	4	4	5	4	2	3	4
c	6	7	2	8	8	3	6	7	8	6	8	8
d	5	2	1	4	5	2	2	6	3	4	4	6
e	4	6	8	5	7	5	8	4	7	7	5	5
f	2	5	7	3	6	7	7	2	5	3	2	3
g	3	4	6	2	2	8	3	3	2	5	6	2
h	1	1	5	1	1	1	1	1	1	1	1	1

The "a" piece divided last 33.3 per cent, and seventh 25 per cent, of the time, that is, it divided relatively more rapidly than in the second filial generation; and the "h" piece divided first 91.6 per cent of the time.

DISCUSSION

Middleton, by repeated selection of slowly and rapidly dividing individuals of *Stylonichia* for hundreds of generations found a difference in fission rate which was maintained long after selection stopped (1915).

Parker (1926) made fast and slow selections in fission rate of *Paramecium aurelia* and found in 200 days a difference of 66 generations.

De Garis (1928) finds in work with *P. caudatum*, that the fission rates of anterior stocks are not invariably higher than those of the posterior, but that heritable differences in fission rate can be brought out by selection of cells of anterior and posterior origin.

My results at normal temperature ranges gave similar indications: that there was no constancy in the relative division of anterior and posterior stocks, but that in animals transferred to lower or higher temperatures there is an apparent inheritance of rate such that the anterior pieces show a faster rate in the higher temperatures while the posterior pieces have the higher rate in the lower ones.

The results suggest an interpretation in terms of Child's gradient theory. The axial gradient in a *Paramecium* has been indicated by various methods, but the evidence from cell lineage presented here is of particular significance since there is less uniformity in cytolysis along the axis of *Paramecium* than with any other ciliate thus far examined (Child and Deviney, 1926). It has been shown for some forms—e.g., *Planaria*—that a rise in temperature up to a certain point accelerates the more active levels to a greater degree than the less active. In view of these facts it may be suggested that the sudden rise of temperature accelerates the physiological activity of the more active levels to a greater extent in the anterior than in the posterior regions of the body. Similarly, the lowering of the temperature retards physiological activity to a greater extent in the anterior regions than in the posterior. The data on the low-temperature series suggest that if cultures were kept at the different temperatures for a longer time, differential acclimatization would obliterate the differences in division rate.

Indications are, as in the work of Middleton and De Garis, that there may be an inheritance of the relative division rates. This in-

heritance of the regional metabolic rates of the original animal may show up in fission products at least to the third generation.

ROBERTSON'S "ALLELOCATALYTIC EFFECT"

In all the following experiments, unless otherwise noted, animals with approximately the same rate and time of division were isolated, allowed to divide until four, eight, or sixteen cells were produced, then all four or eight or sixteen were subjected to the experimental conditions and compared with the similar four or eight or sixteen which were used as a control. Because of their re-isolation to the experimental or control conditions after 1 or 2 days, the lag period is absent. This is in accord with the findings of Greenleaf (1926), who reports that when animals are changed from young cultures to fresh medium no lag is apparent. Robertson (1924) and Myers (1927) report similar experience. In all cases unless otherwise stated, animals were isolated to 24-hour bacterized medium. Four series of experiments were run as follows: (1) cell proximity in small volumes of medium; (2) variation in volume of medium; (3) cell proximity in large volumes of medium; and (4) conditioning of medium, large, and small volumes.

A preliminary series of experiments was carried on by way of introduction. In all these experiments, eight cells, selected as indicated above, were isolated singly, and eight in two's. Washing was accomplished by isolating the animals into 5 drops of fresh bacterized medium, re-isolating to 5 drops of similar medium, and re-isolating to the conditions of the experiment. In this way the conditions of Robertson's experiments are approached. Robertson states (1924, p. 151),

> When two organisms (infusoria) are freed from adherent parent culture fluid by washing and then introduced together into the same restricted volume of fresh culture medium, the rate of reproduction is not merely double that displayed by a single individual similarly treated but some multiple in excess of this varying from 2.5 to 10 or more in different experiments.

He continues,

> In all of my experiments which yielded positive results, the infusoria, prior to the introduction into fresh culture medium, were washed twice in relatively large volumes of fresh culture medium to remove adherent traces of the parent culture liquid.

And further,

> I found as before, that allelocatalytic effect is frequently absent or replaced by actual retardation in unwashed cultures, while it becomes progressively more manifest the more thoroughly the cultures are washed [1924, p. 152].

The results from preliminary experiments each involving the eight progeny of one cell which were subjected to the experimental conditions and were compared with the eight progeny of a similar cell used for control are given in Table II.

In experiments C and F there is a suggestion of allelocatalysis both at the end of 20 and of 44 hours. The others show no indication of allelocatalysis such as has been reported by Robertson, but bear out the findings of Cutler and Crump, Greenleaf, and Myers that

TABLE II

SHOWING THE RESULTS OF THE PRELIMINARY EXPERIMENTS

Transfer	Single						Double					
Hours	0	20	24	28	32	44	0	20	24	28	32	44
Exp. A. 4 drops; unwashed	8	32	57	64	64	182	8	32	49	64	64	179
Exp B. 4 drops; washed once	8	31	32	34	60	115	8	31	32	41	55	118
Exp. C. 2 drops; washed once	8	16	16	31	32	74	8	17	32	32	51	110
Exp. D. 2 drops; washed twice	8	32	32	51	59	111	8	32	32	47	60	106
Exp. E. 4 drops; washed twice	8	16	21	32	63	125	8	16	16	32	32	114
Exp. F. 2 drops; unwashed	8	32	52	64	64	127	8	38	63	64	95	188

animals fail to show acceleration due to the proximity of another cell. Again, contrary to Robertson's findings that successive washings emphasize allelocatalysis, one of the foregoing experiments in which animals were not washed, and one in which they were washed only once gave indications of acceleration; while those in which the animals were twice washed, showed retardation when two animals were placed together, as compared with their isolated controls.

Following the leads from the exploratory experiments, two series were run in 2 drops of bacterized medium. In the first series, the animals were washed once; and in the second, they were isolated unwashed. The results are shown in Table III. Although in the preliminary experiments allelocatalysis was suggested, further similar experiments did not bear out the indications, either in the washed or unwashed animals. Again, contrary to the report of Robertson,

there was no speeding-up of the division rate after the first fission. He reports: "Once a cell division has occurred, however, succeeding cell divisions recur, not at equal but at progressively abbreviated intervals" (1923, p. 82). This very evidently does not hold in the foregoing experiments, which resemble those of Myers in this respect.

TABLE III

Transfer....	Single						Double					
Hours.....	0	12	18	24	36	48	0	12	18	24	36	48
A. Animals Washed Once in Sterile Medium and Transferred to 2 Drops of Bacterized Hay Medium												
	8	16	31	62	64	264	8	14	26	51	51	184
	8	16	32	63	64	254	8	16	32	64	64	255
	8	9	26	32	64	337	8	9	17	32	64	275
	8	16	19	57	64	255	8	16	16	38	64	190
	8	14	15	32	59	232	8	16	16	32	59	234
	8	14	32	32	64	230	8	8	16	32	60	214
	8	8	22	32	64	257	8	8	18	32	64	216
Total....	56	93	117	278	443	1,829	56	87	141	249	426	1,568
Mean....	.....	1.66	3.16	5.53	7.91	32.6	.....	1.55	2.51	5.14	7.60	28.0
B. Unwashed Animals Transferred to 2 Drops of Bacterized Medium												
	8	15	16	32	64	115	8	9	16	32	32	116
	8	16	16	32	64	115	8	16	16	32	50	116
	8	16	16	32	57	129	8	14	15	28	51	124
Total....	24	47	48	96	153	370	24	39	47	92	133	356
Mean....	.....	1.99	2.0	4.0	6.38	15.4	.....	1.62	1.99	3.98	5.54	14.4

Another series of experiments was carried on in an attempt to discover whether the division of an animal in a limited volume of medium would accelerate the rate of division of individuals isolated into the same volume. Washed and unwashed animals were tested. Washed animals were isolated to 2 drops of bacterized medium. At the end of 12 hours the animals were removed, and the slides in which fission had occurred were noted. Five sets consisting of eight animals (each eight were fission products of one animal) were isolated singly into the drops in which fission had recently occurred;

and comparable animals, into those drops where animals had lived without division. The results are shown in Table IV.

Only one reading shows any great difference between the division in the two types of conditions. This difference occurs in the 18-

TABLE IV

SUMMARIZED RESULTS OF EXPERIMENTS ON THE EFFECT OF CONDITIONING MEDIUM ON THE DIVISION RATE OF ANIMALS. (CONDITIONED MEDIUM WAS THAT IN WHICH DIVISION HAD TAKEN PLACE)

A. Unwashed Animals Isolated Singly to 2 Drops of Bacterized Medium in Which an Animal Had Lived 12 Hours and Divided, Compared with Those Isolated to 2 Drops in Which an Animal Had Lived 12 Hours without Division

Medium....	Conditioned						Not Conditioned					
Hours.....	0	12	18	24	36	48	0	12	18	24	36	48
	8	16	58	64	129	345	8	15	32	63	128	391
	8	16	59	64	129	345	8	16	32	64	130	390
	8	20	32	36	64	139	8	21	32	47	128	215
	8	16	32	51	128	209	8	16	32	64	77	133
	8	24	32	48	64	159	8	22	32	46	64	162
	8	36	59	64	160	250	8	38	59	64	159	241
Total....	48	128	272	327	674	1,447	48	128	219	348	686	1,533
Mean....	.....	2.6	5.6	6.8	14.0	31.0	.....	2.6	4.8	7.2	14.3	31.9

B. Animals Washed Two Times in 5 Drops of Sterile Hay Medium and Treated as in A

Medium.....	Conditioned							Not Conditioned						
Hours..... ..	0	4	8	24	28	32	40	0	4	8	24	28	32	40
	8	11	18	64	75	109	183	8	12	18	64	70	109	186
	8	12	17	64	69	100	209	8	11	17	64	72	100	197
	8	12	16	68	78	114	178	8	11	16	69	85	106	182
	8	11	16	68	71	99	186	8	10	16	68	70	105	188
Total.....	32	46	67	264	293	422	756	32	44	67	265	297	420	753
Mean.....	....	1.4	2.9	8.4	9.2	13.2	23.8	....	1.4	2.9	8.4	9.2	13.2	23.8

hour count of the unwashed animals. In this case there is a difference of 53, the medium in which an animal had divided having the higher count. This count depends on an apparent acceleration in but two of the six sets to be compared. In the others there was no observable difference. The count taken 6 hours later, however, shows

a greater number in the sets without previous division. The first difference was due probably to the fact that the higher count was made immediately after division, whereas the lower count was made just before fission, which appears to have come at about this 18-hour period. Obviously, little, if any, importance can be attached to this one observation indicating more rapid division in the conditioned medium.

There was no other noticeable difference in division rate at the beginning or at the end of the experiments, on washed or unwashed individuals. This is not what would be expected on the basis of Robertson's work. His reports indicate that acceleration of division rate should appear after the first division in a limited volume, due to the liberation of an autocatalyst from the nucleus to the pericellular medium at the time of division.

Another attempt to accelerate division rate by the use of conditioned medium was made with cultures of larger volumes. Cultures of 20 drops (approximately 24 drops to 1 cc.) in which an animal had lived and divided were inoculated with isolated unwashed animals and their division compared with that of other comparable animals also unwashed, which were isolated to 20 drops each of fresh bacterized medium. The 20-drop cultures were kept in salt cellars in covered crystallization dishes arranged as for slide cultures. The control medium in all cases was put into the salt cellars at the same time that the experimental medium was set out, and all were put aside for 24 hours before the experimental and control animals were isolated.

Animals isolated into the 20 drops in which one individual had divided and lived (d) and animals isolated into 20 drops of fresh bacterized medium (n.d.) at the end of 18 hours gave the following numbers respectively.

d.	4, 2, 4, 4, 2, 2, 3, 3, 2, 3, 2, 2, 2, 2, 2—41
n.d.	1, 1, 1, 2, 1, 1, 1, 1, 1, 1, 1, 1, 2, 1, 1, 2—19

Thus indications are that an animal living and dividing in 20 drops of medium conditioned it in such a way that an acceleration in the division rate of other individuals isolated into it is marked. The cause of this acceleration is not certain without more experiments.

Apparently in the small volumes, one individual is able to condition the medium sufficiently to cause rapid multiplication; and therefore when one animal is transferred to a small volume of conditioned medium, its rate of division is not faster than that of a similar individual transferred to unconditioned medium. But in large volumes, one individual is not able to condition the medium immediately; and when, therefore, one animal is introduced into a large volume of conditioned medium, division takes place faster than in the case of a similar individual introduced into unconditioned medium.

Greenleaf and Myers in work with conditioned medium used only small volumes, 2–5 drops. Both report a depressed division rate in the conditioned medium, which they interpret as due to the increased toxicity of the old conditioned medium.

The remaining experiments were carried out with hay medium made up with distilled water instead of well water. In these the pH was raised to 8.0 and 8.6 by the addition of M/10 $Na_2 CO_3$. The first two series were run to determine the effect of volume of medium on multiplication of *Paramecium*. One, two, four, and eight individuals, unwashed and unselected, were isolated to 1, 2, and 5 drops of bacterized medium. The results are given in Table V.

The greatest number of animals calculated on the basis of one original animal was found in every case in the 5-drop cultures. Also, the division rate was highest in the one animal culture in 1, 2, and 5 drops at the 48-hour reading. Greenleaf and Myers, both of whom found the same relationship existing between volume and numbers of animals, interpreted the slower rate in small volumes and with larger numbers of individuals as due to the accumulation of excretion products.

It seemed possible that the presence of bacteria in the medium may be responsible for the accumulation of toxic substances and that using sterile hay medium might help to bring out Robertson's allelocatalysis. H. B. Yocum, working with a small ciliate, *Oxytricha*, isolated them to sterile medium of volumes of 4–10 drops (100 drops to 1 cc.) without previous washing, and found that at the end of 24 hours there had been more fission in the smaller volumes than in the larger. His results appear to contradict those of Greenleaf.

In my experiments on this point, sterile hay made up with dis-

TABLE V

SHOWING THE NUMBERS OF INDIVIDUALS PRODUCED IN 1, 2, AND 5-DROP CULTURES, CONTAINING ORIGINALLY ONE, TWO, FOUR, AND EIGHT ANIMALS, UNWASHED AND TRANSFERRED TO BACTERIZED ALKALINIZED MEDIUM

Cultures....	1 Drop				2 Drops				5 Drops			
Hours.....	0	9	24	48	0	9	24	48	0	9	24	48
One Animal												
	1	1	2	2	1	1	2	4	1	1	2	4
	1	1	2	4	1	1	2	4	1	1	2	4
	1	1	4	4	1	1	2	2	1	1	2	4
	1	1	2	4	1	2	4	4	1	1	2	4
	1	1	2	4	1	1	2	4	1	2	4	8
	1	1	2	4	1	2	4	6	1	2	4	8
	1	1	2	4	1	1	2	4	1	1	2	4
	1	1	2	4	1	1	2	4	1	1	2	4
Total....	8	8	18	30	8	10	20	32	8	10	20	40
Mean....	.....	.....	2.25	3.75	.....	1.25	2.50	4.0	.....	1.25	2.5	5.0
Two Animals												
	2	2	4	4	2	2	4	4	2	2	4	8
	2	2	4	8	2	2	4	4	2	2	4	8
	2	2	2	8	2	2	4	4	2	4	8	12
	2	3	4	8	2	2	4	4	2	2	4	8
	2	2	4	8	2	2	4	6	2	3	8	8
	2	2	5	5	2	2	4	5	2	2	4	8
	2	2	4	4	2	2	4	4	2	3	8	12
	2	2	4	4	2	2	5	8	2	3	5	8
Total....	16	17	31	49	16	16	33	39	16	21	45	72
Mean....	.....	1.03	1.93	3.06	.....	.....	2.06	2.14	.....	1.31	2.81	4.5
Four Animals												
	4	4	6	6	4	4	8	8	4	4	8	14
	4	4	6	8	4	5	10	10	4	5	8	16
	4	4	8	8	4	4	8	8	4	4	8	8
	4	4	8	8	4	4	9	9	4	5	8	16
	4	4	8	8	4	4	8	8	4	5	9	16
	4	4	8	8	4	5	10	14	4	4	8	12
	4	4	8	8	4	4	8	8	4	4	8	14
	4	5	9	12	4	4	8	8	4	4	8	12
Total....	32	33	61	66	32	34	69	73	32	35	81	108
Mean....	.....	1.03	1.93	2.09	.....	1.06	2.15	2.28	.....	1.09	2.47	3.37

TABLE V—*Continued*

Cultures....	1 Drop				2 Drops				5 Drops			
Hours.....	0	9	24	48	0	9	24	48	0	9	24	48
	Eight Animals											
	8	9	16	16	8	8	16	16	8	9	16	30
	8	8	12	16	8	8	16	16	8	9	16	16
	8	9	15	16	8	8	16	16	8	10	20	32
	8	10	16	16	8	8	16	16	8	9	16	16
	8	8	10	16	8	8	15	16	8	9	18	32
	8	9	14	16	8	8	16	16	8	8	16	16
	8	9	13	16	8	8	16	18	8	8	16	16
	8	9	15	16	8	8	15	16	8	9	16	16
Total....	64	71	111	128	64	64	126	130	64	71	134	174
Mean....	.....	1.29	1.73	2.0	.....	.....	1.96	2.03	.....	1.29	2.12	2.71

tilled water was used. The pH of this was raised to that of the old culture medium by the addition of 5 drops of M/10 sodium carbonate solution to 25 cc. of medium. *Paramecia* were isolated to 1 and

TABLE VI

SHOWING THE NUMBER OF ANIMALS PRODUCED IN 24 HOURS BY INDIVIDUALS TRANSFERRED SINGLY TO 1 AND 5 DROPS OF STERILE MEDIUM

Medium.....	1 Drop		5 Drops	
Hours.....	0	24	0	24
	8	24	8	21
	8	23	8	23
	8	27	8	19
	8	26	8	21
	8	22	8	19
Total.....	40	122	40	103
Mean.....	.....	3.05	.....	2.57

5 drops and were counted 24 hours later. The progeny of the two cells with similar time and rates of division were used for the experiment and control. Only five sets were compared, but the difference is statistically significant. See Table VI.

There is an indication of greater division rate in the smaller vol-

umes here, but the numbers are small and the difference does not nearly approach that of Robertson's experiments. It may be that *Paramecia* divide too slowly to bring out the effect especially when a sterile culture medium is used. With the inoculation of animals comes the introduction of bacteria which multiply much faster than the *Paramecia* and thus do away with the sterile condition of the medium. It is possible that there is a difference in the rate of growth of the introduced bacteria in proportion to volume or that the lower

TABLE VII

SHOWING DIVISION RATE OF SETS OF FOURS TRANSFERRED SINGLY, IN TWOS, AND IN FOURS TO 20 DROPS BACTERIZED MEDIUM. WASHED TWICE IN BACTERIZED, AND ONCE IN ALKALINIZED MEDIUM

Transfer	One			Two			Four		
Hours	0	24	48	0	24	48	0	24	48
	4	5	9	4	5	16	4	8	18
	4	4	9	4	5	10	4	5	12
	4	5	9	4	6	13	4	6	16
	4	4	11	4	5	20	4	7	16
	4	4	9	4	6	10	4	6	14
	4	8	16	4	8	17	4	8	24
	4	6	12	4	8	24	4	8	24
	4	7	14	4	8	16	4	8	16
	4	7	16	4	14	24	4	14	24
	4	9	10	4	8	12	4	10	14
Total	40	59	115	40	74	162	40	80	178
Mean	.....	1.47	2.87	.....	1.85	4.05	.....	2.0	4.45

concentration of bacteria in the larger volume acts as a limiting factor.

Because of the evidence of retardation in similar volumes of the earlier experiments it seemed possible that the volumes used might be entirely too small. Therefore, in the next group of experiments, volumes of 20 drops were used. One, two, and four animals were isolated to 20 drops of bacterized alkalinized medium having a pH of approximately 8.6 in place of the 7.8–8.0 usual in stock cultures. The animals were washed twice in bacterized medium and once in alkalinized medium. Animals with approximately the same rate and time of division, allowed to divide two times, were used. Counts were made after 24 and 48 hours. See Table VII.

In these experiments there is very evidently something approaching the allelocatalytic effect. It may be that the smaller volumes were too small for an animal as large as *Paramecium caudatum* and that the retardation due to excretion products became noticeable almost immediately; or that the autocatalyst, if Robertson's explanation is correct, was concentrated sufficiently in the small volume when only one cell was present, and became too concentrated when more cells were introduced.

TABLE VIII

SHOWING RATE OF DIVISION OF SETS OF FOURS TRANSFERRED SINGLY, IN TWOS, AND IN FOURS IN INFESTED CULTURES OF 20 DROPS OF BACTERIZED MEDIUM

Transfer	One			Two			Four		
Hours	0	24	48	0	24	48	0	24	48
	4	8	8	4	8	8	4	8	8
	4	8	8	4	8	8	4	8	8
	4	8	8	4	8	8	4	8	8
	4	8	8	4	8	8	4	8	8
	4	8	10	4	8	8	4	8	10
Total	20	40	42	20	40	40	20	40	42
Mean	.....	2.0	2.1	.....	2.0	2.0	.....	2.0	2.1
	4	8	.....	4	8	.....	4	8	.....
	4	8	.....	4	8	.....	4	8	.....
	4	7	.....	4	8	.....	4	10	.....
	4	7	.....	4	8	.....	4	8	.....
	4	8	.....	4	8	.....	4	8	.....
Total	20	38	.....	20	40	.....	20	42	.....
Mean	.....	1.9	.....	.....	2.0	.....	.....	2.1	.....

Another interesting point was noticed in this series of experiments. There were five sets, similar to those reported above, which at the end of 24 hours were noticeably infested with a small ciliate. In these the rate of division was nearly or quite independent of the initial seeding, which means that the so-called "allelocatalytic effect" was nearly or completely obliterated. Table VIII gives the data on infested cultures. Another set of contaminated cultures again gave no suggestion of Robertson's allelocatalysis. Evidently there is an accumulation of waste products or of autocatalyst just as in the 2-drop cultures.

Since the first group of animals tested in the larger volumes of medium was washed three times instead of the usual two times, it

TABLE IX

SHOWING THE EFFECT OF DOUBLE WASHING AND TRANSFER TO 20 DROPS OF ALKALINIZED BACTERIZED MEDIUM

No.	One			Two			Four		
Hours	0	24	48	0	24	48	0	24	48
	4	6	21	4	8	30	4	8	38
	4	7	12	4	8	16	4	10	36
	4	6	25	4	7	34	4	8	37
	4	8	23	4	8	30	4	8	52
	4	6	22	4	8	34	4	8	40
	4	5	28	4	5	34	4	7	42
	4	7	25	4	8	47	4	9	56
	4	6	9	4	8	22	4	8	33
	4	7	18	4	8	32	4	8	38
	4	6	16	4	7	18	4	8	20
	4	6	14	4	8	16	4	8	23
	4	5	22	4	8	17	4	8	24
	4	5	14	4	6	18	4	8	20
	4	6	28	4	7	34	4	7	40
Total	56	86	277	56	104	382	56	113	493
Mean	.....	1.53	4.94	.....	1.85	6.81	.....	2.01	8.80

TABLE X

SHOWING THE EFFECT OF DOUBLE WASHING AND TRANSFER TO 20 DROPS OF UNALKALINIZED BACTERIZED MEDIUM

No.	One		Two		Four	
Hours	0	24	0	24	0	24
	4	14	4	16	4	16
	4	16	4	16	4	16
	4	15	4	16	4	16
	4	20	4	28	4	30
	4	18	4	16	4	28
	4	14	4	24	4	30
	4	19	4	22	4	26
	4	21	4	26	4	26
	4	16	4	27	4	30
	4	18	4	23	4	31
	4	19	4	24	4	26
	4	21	4	26	4	30
Total	48	211	48	264	48	305
Mean	.....	4.39	.....	5.5	.....	6.35

seemed possible that the extra washing was responsible for the results obtained. To test this, fourteen sets of four each were isolated as above, after being washed twice in bacterized hay medium. They were isolated into 20 drops of alkalinized hay medium (see Table IX). The results show that the third washing in the case of the other group was not responsible for the acceleration of the division of the four animals transferred together.

The next question arising was whether the alkalinized medium was responsible. Accordingly, animals were twice washed in bacterized medium and isolated to 20 drops of bacterized hay medium

TABLE XI

SHOWING THE EFFECT OF DOUBLE WASHING AND TRANSFER TO 1 DROP OF ALKALINIZED BACTERIZED MEDIUM

No.	One			Two			Four		
Hours	0	24	48	0	24	48	0	24	48
	4	7	20	4	5	12	4	6	12
	4	7	14	4	4	12	4	4	8
	4	7	20	4	6	22	4	7	16
	4	5	15	4	7	16	4	6	14
	4	7	17	4	7	18	4	8	16
	4	4	16	4	4	19	4	4	20
Total	24	37	102	24	38	99	24	35	86
Mean	.....	1.54	4.26	.....	1.54	4.11	.....	1.54	3.59

with a pH of 6.8, which is lower than that of the parent-culture of 7.8 to 8.0. The results are given in Table X and show that the alkaline medium used in other experiments was not responsible for the acceleration of division when two and four animals are inoculated in 20 drops.

As another check on the possible accelerative action of three washings and transfer to alkalinized medium, one, two, and four animals after two washings in bacterized medium, followed by one in alkaline medium, were transferred to 1 drop of bacterized alkalinized medium with a pH of 8.6. Just as in the early experiments with small volumes and lower pH, the single animals divided at least as fast as when two were placed together, and somewhat faster than did the four. Table XI gives the summarized results.

Again there was no evidence of the allelocatalytic effect, but the results entirely support earlier experiments with smaller volumes. It seems that the inability to obtain any evidence of allelocatalysis previously has been due to the use of too small volumes of medium in proportion to the size of the animals.

On the other hand, animals treated in the same way and introduced into 40 drops of medium showed some acceleration when four were inoculated together, but none at all when two were introduced

TABLE XII

ANIMALS WASHED THREE TIMES AND TRANSFERRED SINGLY, IN TWOS, AND IN FOURS, TO 40 DROPS ALKALINIZED, BACTERIZED MEDIUM

Transfer	One			Two			Four		
Hours	0	24	48	0	24	48	0	24	48
	4	8	16	4	8	16	4	8	16
	4	8	16	4	8	16	4	8	16
	4	8	16	4	8	16	4	9	19
	4	4	4	4	4	4	4	4	4
	4	4	4	4	4	4	4	4	4
	4	4	4	4	4	4	4	4	8
	4	8	16	4	8	16	4	12	18
	4	8	16	4	8	16	4	10	16
	4	8	16	4	9	16	4	10	16
	4	8	32	4	8	32	4	16	40
	4	8	22	4	8	22	4	8	24
Total	44	76	162	44	77	162	44	93	181
Mean	.....	1.75	3.69	.....	1.75	3.69	.....	2.11	4.11

and examined after 24 hours or after 48 hours. Apparently, two animals were not able to condition 40 drops sufficiently to show an increased division rate over cultures containing one animal. More work is necessary on this point. Data are given in Table XII.

DISCUSSION

Since Robertson reported allelocatalysis, and Yocum confirmed him in so far as his experiments cover the same ground as those of Robertson, and since the animals used by both were relatively small, it seemed possible that the volume relations of medium and animals might be responsible for the positive results. My own experiments seem to demonstrate that such is the case. In large volumes of cul-

ture medium an effect approaching allelocatalysis was noticed; while in small volumes the effect, if originally present, was obliterated as the result of crowding. A comparison of Tables VII, XI, and XII, the results of which are most nearly comparable, brings out these points computed on the basis of one animal.

A statistical examination of the numbers produced in 20-drop cultures initially containing one and four individuals shows that there are only 2 chances in 10,000 of obtaining so great a deviation in either direction by random sampling. When the data for the 40-drop cultures are examined statistically, they show 5 chances in 100 of obtaining so great a deviation in either direction by random sampling. Such results are usually considered to have statistical significance. After 24 hours the animals transferred singly to 1 drop

Animals	1		2		4	
Hours	24	48	24	48	24	48
1 drop	1.54	4.26	1.54	4.11	1.54	3.59
20 drops	1.47	2.87	1.85	4.05	2.00	4.45
40 drops	1.75	3.69	1.75	3.69	2.11	4.11

had a slightly faster rate than those transferred to 20 drops. Cultures initially containing two or four individuals showed a greater number of divisions at the end of 24 hours in the 20-drop cultures. In the 48-hour period the one individual in 1 drop divided faster than the one animal in 20 drops; but when two or four individuals were inoculated into 1 drop, the rate was slower in the two- and still slower in the four-animal cultures; whereas when they were put into 20 drops, the rate was faster in the two- and still faster in the four-animal cultures, as compared with the division rate of one animal in the same volumes of culture medium. In the 40-drop cultures the two-animal cultures showed no acceleration, but the four-animal cultures showed a faster rate. In the 1-drop cultures the rate became progressively slower as the initial numbers of animals were increased, but in the 20 drops it became progressively faster in 48 hours. The data for the 24-hour period in the single-drop cultures suggest not so much retardation due to the accumulation of toxins as a conditioning of the medium by one individual sufficient to cause division

to take place at the same rate as in cultures of the same size initially containing two or four individuals. In the 20-drop cultures apparently the one individual is not able to condition the medium sufficiently in 48 hours to enable it to divide at as high a rate as in the cultures containing the larger numbers of animals. In the 40-drop cultures neither one nor two animals are able to condition the medium completely. The observed rate is higher for single animals in 40 drops than in 1 drop. This may be due to a temperature difference, since the 1-drop and 20-drop experiments were run in winter and the 40-drop experiments were run in summer, or to a difference in the physiological condition of the animals. Also, when Tables VII and VIII are compared, it is quite evident that the retardation in 20-drop cultures initially containing one animal is less when cultures contain small ciliates. Apparently the other ciliates condition the medium and at first benefit the isolated *Paramecium*. The ratio of volume of medium to volume of animal is smaller in these cultures than in those cultures which show allelocatalysis.

These observations led to a comparison of the volume relations of medium and animals used by other investigators. It was found that in all cases where acceleration was reported or indicated the volume relations of medium and animals are similar, the ratio being over 200,000; but in those cases where no acceleration was noticeable, the ratio of volume of medium to volume of animal is smaller, under 100,000 or very much larger than 200,000. These comparisons are summarized in Table XIII.

Greenleaf's volume relations with *Paramecium caudatum*, *P. aurelia*, and *Pleurotricha lanceolata* were similar to those ratios in which acceleration was noticed or indicated. He found no evidence of the allelocatalytic effect, but this may have been covered by his method of recording the mean numbers produced for 5-day periods.

Cutler and Crump also report no acceleration in division rate when more than one animal is introduced into a limited volume. They used volumes of 0.5–9.0 cu. mm. A comparison of numbers of animals produced in each volume initially containing one, two, three, and four animals does not show differences of statistical significance; but when averages of all volumes from 0.5 to 2.5 cu. mm. for one and two, one and three, and one- and four-animal cultures are taken

together and examined statistically by "Student's" method for paired experiments, there are respectively 4 chances in 100, 2 chances in 100, and 15 chances in 1,000 of obtaining so great a deviation in either direction by random sampling (1923, p. 881). When volumes up to 2.5 cu. mm. were considered—a medium-animal ratio of ap-

TABLE XIII

SHOWING THE VOLUME RELATIONS OF ANIMALS AND CULTURE MEDIUM USED IN WORK ON ALLELOCATALYSIS IN PROTOZOA*

ANIMAL	SIZE IN MM.	VOL. IN CU. MM.		VOL. MEDIUM / VOL. ANIMAL
		Animal	Medium	
Enchelys farcimen† (Robertson)	0.07×0.05	0.000175	80.0	457,142
Colpoda cucullus† (Robertson)	0.08×0.06	0.000288	80.0	277,777
Colpidium colpoda (Cutler and Crump)‡	0.044×0.01	0.0000044	1.0	227,272
†			2.5	568,181
			8.5	1,931,745
Stylonychia pustulata (Greenleaf)	0.25×0.10	0.0025	50.0	20,000
Paramecium‡ *caudatum* (Greenleaf)	0.25×0.065	0.001056	1,000.0	946,960
Oxytricha pellonia(?) (Yocum) †	0.08×0.032	0.0000819	40.0	488,403
Paramecium caudatum (Myers)‡	0.25×0.065	0.001056	100.0	94,696
†			800.0	757,860
Paramecium caudatum (Petersen)	0.263×0.058	0.000847	83.3	98,347
†			833.3	983,470
			1,666.6	1,023,140
Uroleptus mobilis (Calkins)	Size of drop is not given			

* Dimensions of *Enchelys farcimen* and *Colpidium colpoda* were obtained from Cutler and Crump, 1923; *Colpoda cucullus*, from Robertson, 1927; *Stylonychia pustulata*, *Paramecium caudatum* and *Oxytricha*, from Pratt, *Manual of the Common Invertebrate Animals*, and checked by Conn, 1905. *Paramecium* used in my experiments were measured directly. The species of *Oxytricha* used by Yocum is not given; but because the small size was emphasized, the dimensions of *Oxytricha pellonia* were used in the foregoing calculations.

† Acceleration when two individuals were present.

‡ See discussion.

proximately 500,000—the data indicate that one-animal cultures in the first 24 hours had a lower rate of fission than two-, three-, or four-animal cultures and that the differences between 1 and 2, 1 and 3, and 1 and 4 are statistically significant.

The results of Cutler and Crump show that the rate of division of single animals increases with volume of medium. They also find that the effect of two organisms in a given volume gives a more rapid

rate of division than when only one is present for half that volume. The first looks like contamination or food effect; and the second, like allelocatalysis. For reasons given above, my experiments cannot be used to throw light on this point.

Myers reports no acceleration due to the contiguity of two or more cells. Data given in column 4, representing mean hours to the first division of the five lines, of his Tables 5, 6, and 7, where one, two, four, and eight individuals were transferred to 16 drops of medium, either fresh or after standing 1 or 2 days, suggest a different interpretation from that given. In the 1-day infusion the data suggest that when one individual was inoculated into 16 drops, three of the five lines, averaged together in each case, had divided at the 6-hour period and two at the 12-hour period, making a mean of 8.4 hours to the first fission. When two and four animals were treated similarly, four lines divided at the 6 hours and one at 12 hours, making a mean of 7.2. When eight individuals were transferred together under similar conditions, all five lines divided before the 6-hour period. In 2-day infusions, the data appear to show that three of the five lines of isolated individuals had divided before the 6-hour observation and two others before the 12-hour period, giving a mean recorded time of 8.4 hours. The cultures originally containing two, four, and eight animals had all divided in the first 6 hours. Myers states that "if the initial volume of fluid is large, as when 16 drops are used, fission begins at the same time, whether one, two, four or eight individuals are introduced; the time to the first fission does not differ." Apparently he does not regard changes from 6.0 to 8.4 hours as being significant, although this is an increase of 40 per cent. These relations from Myers' paper are summarized in Table XIV, this article. Of course, the technique used and the system of making observations in 6-, 6-, and 12-hour periods may require greater differences before they become significant and may do away with the indicated allelocatalytic effect. In the case of the fresh medium, the increase in the lag period when eight animals are introduced suggests a food relation, such that there is insufficient food present in the fresh medium to support eight individuals. However, here again, the cultures seeded with two and four animals divide before those started with only one.

In Myers' work the length of life in the cultures started with one animal was longer than any of the others. Robertson recognized this fact, and also that the ultimate effect of the introduction of a second individual is always a retardation of the mean reproductive rate. The mean division rate from the time of the first division to the time of maximum numbers in a culture would be expected to be slower when eight individuals show signs of crowding or possibly deficient food supply early in the life of the culture, which is in accord with Robertson's findings. But in Myers' one-, two-, and four-individual cultures, the ratio of division to the time of maximum is greater when four individuals are introduced than in cultures starting with only two. The allelocatalytic effect is not shown in the suc-

TABLE XIV

DATA FROM MYERS' TABLES 5, 6, AND 7. SHOWING THE NUMBERS OF HOURS TO FIRST DIVISION IN ANIMALS TRANSFERRED IN GROUPS OF 1, 2, 4, AND 8–16 DROPS OF FRESH, 1-DAY AND 2-DAY INFUSIONS. OBSERVATIONS MADE AT 6-, 6-, AND 12-HOUR PERIODS

Ratio of Individuals to Drops	Fresh Infusion	1-Day Infusion	2-Day Infusion
1:16	27.6	8.4	8.4
2:16	24.0	7.2	6.0
4:16	25.2	7.2	6.0
8:16	56.4	6.0	6.0

cessive 12-hour periods when observations were made on one, two, four, and eight individuals transferred to 16 drops of 2-day medium. Apparently the acceleration shown in the 6-, 6-, and 12-hour observation periods as given in Table 7 of Myers' paper is smoothed off if the 6-hour observation periods are disregarded.

In my own experiments, with 20 drops at the end of 24 hours there was acceleration in the cultures originally containing two and four animals, greater in the case of the four-animal culture, but at the end of 48 hours there was a retardation in the four-animal culture. In the 40-drop cultures there was acceleration at the end of 24 and 48 hours in the four-animal cultures.

Apparently, when one individual was introduced into small volumes of medium, it was able to condition the medium in a sufficiently short time to cause division to proceed as rapidly as when two or more *Paramecia* are transferred together into similarly small vol-

umes; but when one individual is introduced into large volumes, it is not able to condition the medium, and a retardation in division is noticeable as compared with similar cultures containing more individuals run simultaneously.

Indications are, however, that with *Paramecium caudatum*, unlike Robertson's conclusions with *Enchelys farcimen* and *Colpoda cucullus*, the substance which caused the acceleration is not in the nucleus alone, or at least it is not freed at the time of division alone. On the contrary, with *Paramecia* from young cultures transferred to fresh bacterized medium, conditioning of the medium begins at the time the individuals are isolated into it, and continues as long as they are there. In my experiments there was no evidence of a progressive shortening of the division rate of animals in a given volume, as Robertson claims.

SUMMARY

1. When the two daughter-cells of *Paramecium* were suddenly subjected to high temperatures, 26°–30° C., the anterior cell always divided first; when they were subjected to low temperatures, 13°–17° C., the posterior cell always divided first.

2. In the division of cells of the second and third filial generation in high temperatures the most anterior usually had the highest rate and the most posterior the lowest rate. When the animals were subjected to low temperatures, the second and third generation usually showed the most posterior cells with the highest rate, and the most anterior cells with the lowest rate of division.

3. At normal temperatures, 18°–22° C., the relative rate of division varied.

4. When animals were isolated singly and in two's into small volumes of medium, 2 or 4 drops, there was no noticeable acceleration in division in the two-animal cultures. Washing had no effect on the division rate.

5. Animals, washed and unwashed, introduced singly into small volumes of conditioned medium showed no acceleration in division rate over the control animals in unconditioned medium. When they were isolated to 20 drops of conditioned medium, division was apparently accelerated.

6. Unwashed animals introduced singly into varying volumes of sterile medium showed greater division in the smaller volumes within a given time.

7. When unwashed animals in groups of one, two, four, and eight were isolated to 1, 2, and 5 drops of bacterized medium, the greatest number of divisions took place in the 5-drop cultures originally containing one animal.

8. Washed or unwashed animals introduced singly, in two's, and in four's to 20 drops of bacterized medium showed the highest division rate in the four-animal cultures and the lowest in the one-animal cultures.

9. When animals were isolated into bacterized medium infested with ciliates, there was no acceleration in division in the two- and four-animal cultures.

10. When animals were isolated to 40-drop cultures, they divided more rapidly in the four-animal cultures than in the one- and two-animal cultures, which were about equal. After 48 hours, acceleration was noticeable in both the two-and four-animal cultures.

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INTEGUMENTARY PIGMENTATION IN THE FROG, RANA PIPIENS, DURING METAMORPHOSIS, WITH ESPECIAL REFERENCE TO TAIL-SKIN HISTOLYSIS

(Two plates and one figure)

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INVESTIGATIONS upon the specificity of integument in amphibians have been confined to a comparatively recent date. Considerable experimental work has been reported within the last few years, the greater part of which has been upon the salamander. In this regard the findings of Weigl (1913) and Uhlenhuth (1917) are of interest. The former found that homoplastic transplants of salamander (*Salamandra maculosa*) larvae integument retained their specific pigmentary characteristics through metamorphosis. Uhlenhuth, working with *Amblystoma punctatum*, made similar observations and concluded that two factors were responsible for pigmentation: one within the skin itself, and one which was within the blood stream and which he calls a "metamorphosis factor." Winkler (1910) made homoplastic, integumentary transplants on *Hyla* and found that belly and back skin retained their pigmentary characters indefinitely.

A more recent work in this connection has been reported by Cole (1922), working with *Rana clamitans* and *R. catesbeiana* larvae. He noted that tail, back, and belly skin transplanted to any region of the body maintained its individual pigmentary characteristics indefinitely during the larval life of the animal.

It seemed worth while that a study be made of pigmentary reactions in integumentary grafts as they occur during metamorphosis. Series of operations were accordingly planned which involved the reciprocal transplantation of larval skin grafts from areas known to differ widely in pigmentation pattern following metamorphosis. In this way light could be thrown on the possible specificity or nonspecificity of integumentary pigmentation. If the integument is nonspecific in this respect, then we should expect transplanted skin grafts to assume the pigmentation, during metamorphosis, typical

of the region to which they had been transplanted. Conversely, if the integument is specific, we should expect skin grafts to develop the same pattern and type of pigmentation during metamorphosis in the foreign location as they would have developed if not transplanted.

Another question might well be raised regarding the mechanism of integumentary histolysis in metamorphosing tadpoles. Several theories have been formulated in an attempt to explain the factors responsible for inducing tail atrophy. Among the most important is that of Barfurth (1887), who maintained that the growth of the pygostyle initiates the process of tail atrophy by occluding the blood vessels at the base of the tail. Bataillon (1891) likewise thought that the rapidly growing pygostyle caused a change in the distribution of the blood supply to the tail. Dr. O. M. Helff of this laboratory, in unpublished investigations, has recently removed the pygostyle from normal tadpoles and finds no appreciable difference in the rate at which the tail atrophies during metamorphosis. In no case was normal atrophy delayed. Morse (1918) suggested that the atrophy is probably induced by a condition of acidosis in the muscle tissue of the tail, the causation of which was problematic.

Interesting as these investigations are, relatively few observations have been made regarding the mechanism of integumentary histolysis. Helff (1926) produced integumentary histolysis in tadpoles by transplanting atrophying gill and tail muscle beneath opercular skin grafts. This author suggested the possibility that all atrophying or degenerating tissue in metamorphosing larvae may be capable of initiating integumentary histolysis when in contact with the integument.

In this connection it seemed profitable that a study be made regarding the specificity of histolytic reactions in tail and back skin grafts as they occur during metamorphosis. Series of transplantation experiments were planned which involved an interchange of larval skin grafts from the tail and back. If tail skin is specific as regards histolysis, we should expect it to undergo disintegration during metamorphosis, when transplanted to a foreign location. Should the tail skin be non-specific, however, in this respect, and subject to histolysis because of its association with other structures of the tail,

we should not expect it to undergo disintegration during metamorphosis when transplanted to foreign locations.

The present work was carried out at the Iowa Lakeside Laboratory during the summer of 1928. I am indebted to Dr. O. M. Helff for suggesting the problem and for his valuable suggestions and criticism during the course of the work.

MATERIAL AND METHODS

The species of frog larvae used in this investigation was that of *Rana pipiens*. The larvae were collected from a small pond near Spirit Lake, Iowa, during the months of June and July. The animals were taken to the laboratory, placed in large battery jars, and fed daily with fresh *Spirogyra*. From this common stock, normal tadpoles of a uniform size were selected for the various experiments. The individuals selected for use ranged from 60 to 100 mm., body length, with hind limbs varying between 4 and 15 mm. The animals used were normal tadpoles and showed no signs of metamorphosis for at least 2 weeks following the operations.

All experiments necessitated the use of an anesthetic which would render the animal inactive for some time. For this purpose a 0.05 per cent aqueous solution of chloretone was employed. The time required for anesthetization by this method varied from 5 to 10 minutes, depending somewhat upon the size of the animal and the temperature of the chloretone solution. The animals were allowed to remain in the weak chloretone solution for a few minutes after they failed to respond to any mechanical stimuli. Animals so anesthetized remained inactive for at least 2 hours, thus giving adequate time for operative work and postoperative adhesion of grafts and cut edges.

The usual method of skin transplantation was employed. Following the anesthetization, the animal was placed upon a damp cloth under a binocular microscope. An incision was first made, by means of a fine pair of dissecting scissors, around the area of skin to be transplanted. The skin was then stripped from the underlying tissue and transplanted to another region of the body.

With the exception of Series C, autoplastic transplants were made consisting of an interchange of skin grafts from two regions of

the same animal. Figure A illustrates the regions from which the transplants were taken and the areas to which they were grafted. Following the operation, the animals were placed in a Petri dish, partially filled with water, in such a way that the operated areas were exposed to the air. One-half hour of such exposure was usually adequate to allow sufficient adhering of the grafts so that the animals could be immersed completely in water. Daily observations were then made of each individual in respect to the healing process, pigmentary changes, and the progress of metamorphosis. A complete record was maintained for each animal.

RESULTS

SPECIFICITY OF INTEGUMENTARY PIGMENTATION DURING METAMORPHOSIS

1. *Types of skin transplantations.*—Series A consisted of autoplastic skin transplants in which skin from the back was interchanged with a similar graft from the right side (Fig. A, *B*, *S*). Care was

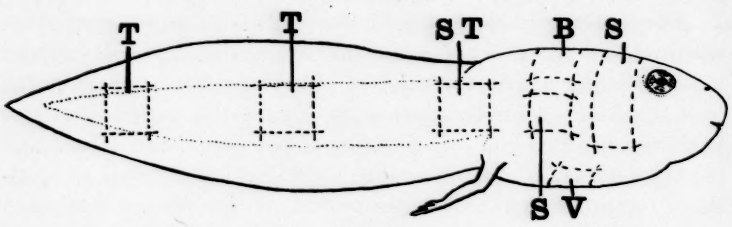


FIG. A

taken to obtain skin grafts from the regions known to develop definite pigment patterns during metamorphosis. Furthermore, the grafts were so placed that their original anteroposterior orientation was not changed. Eighteen cases were obtained, ten of which were permitted to metamorphose completely, the remaining eight being fixed for histological study at various stages of metamorphosis. A second series (Series B) consisted of removing a section of skin about 1 cm. wide, extending from the mid-dorsal to the mid-ventral line. This graft was then rotated 180°, so that the original dorsal or back portion was in contact with the belly and vice versa (Fig. A, *S*, *B*).

Of this type of operation, twelve animals underwent complete metamorphosis, while six were fixed at different stages for histological study.

In consequence of several difficulties encountered in making reciprocal, autoplastic transplants from back to belly, a third type of operation (Series C), consisting of homoplastic skin transplants from the foregoing regions, was carried out (Fig. A, *B*, *V*). The operation consisted of removing a section of skin from the back of an animal and replacing it with a similar graft taken from the belly of another, and in turn transplanting the section taken from the back of the former to the belly of the latter. Eleven animals in this group were allowed to metamorphose, while several were utilized for histological study.

The animals used for the foregoing series were normal tadpoles in every respect, ranging from 70 to 80 mm. in length. The time required for the grafts to heal varied somewhat, depending on the size of the graft, large grafts requiring a slightly longer period, due undoubtedly to the larger wound-area present. Normally 8–10 days were found sufficient for the process to take place. In the course of the healing process it was noted that the grafts appeared slightly darkened for at least 2 days following transplantation. At the end of this period, however, the grafts quickly regain their normal healthy color. However, it was noted that a slightly darkened area persisted around the border of the graft. Upon examination this was found to be due to several types of pigment cells which had been carried into that region along with the migration of epithelial cells which cover it. This darkened area remained for a considerable period of time, often long after metamorphosis.

2. *The development of characteristic pigment patterns in grafts.*—The foreign locations of the various skin grafts in no case effected their pigmentation pattern during metamorphosis (Plate I, Figs. 1, 2, 3, 4, 5). Of the forty-one cases obtained, all retained their specific pigmentary characteristics. Skin transplanted from back to side, or side to back, as in Series A, developed normal spotting characteristic of the region from which they had been taken. As far as could be determined, the number of spots, their intensity of color, and orientation were identical with those of normal integument. Skin trans-

PLATE I

FIG. 1.—Side graft transplanted to back. *G*, graft; *B*, back skin; *P*, pigment spot, showing migration of pigment spot into graft.

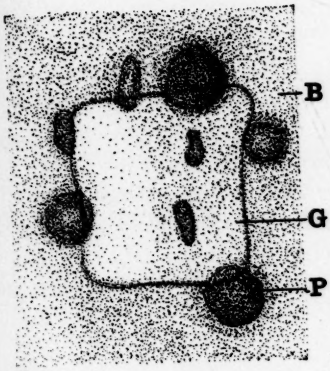
FIG. 2.—Belly graft on back. *G*, graft; *B*, back skin; *P*, pigment spot, showing failure of spots to migrate into graft.

FIG. 3.—Back graft on belly. *G*, graft; *V*, belly skin; *P*, pigment spot. No pigment migration from graft.

FIG. 4.—Back graft to side. *G*, graft; *S*, side skin; *P*, pigment spot. Very slight pigment migration.

FIG. 5.—Back-skin graft on atrophying tail. *G*, back-skin graft; *T*, tail integument; *P*, pigment spots. Showing normal development of back skin on atrophying tail.

PLATE I



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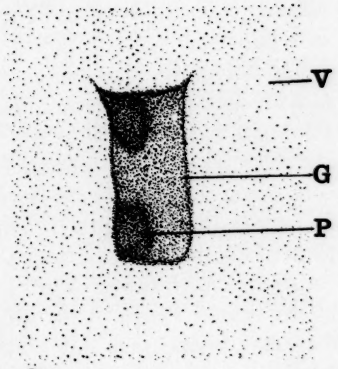

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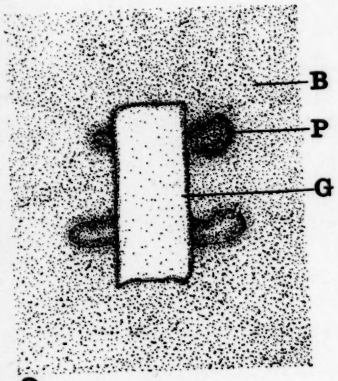

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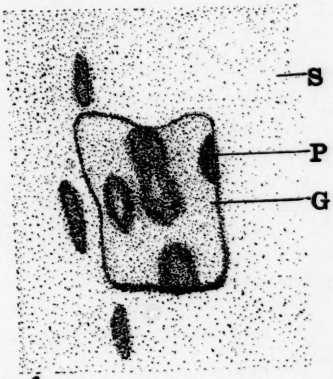

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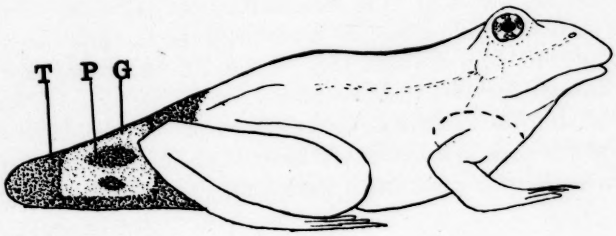

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planted from the back to belly produced its normal spotting in the new position.

Histological examination of the various grafts gave no evidence of a marked decrease or increase in the amount of pigment. A slight variation was noted, however, in Series A, and to a lesser extent in the other series. This consisted of a general tendency of the pigment composing the spots of the normal host skin to migrate slightly into the graft. Plate I, figure 1, represents a graft from the side transplanted to the back, in which this occurred. This was a frequent occurrence in this type of graft and was found to be true in at least 75 per cent of the cases. It was only in this type of graft, however, that such pigmentary migration was observed. In the grafts taken from the back and transplanted to the side, for example, no such migration occurred from either the graft or host skin (Plate I, Fig. 4). The same holds true for back to belly and for belly to back grafts (Plate I, Figs. 3, 2). Upon close examination it was found that such migration took place in the epithelial layer of the skin only, and was therefore quite superficial. By means of a sharp knife it was possible to peel off the layer of epithelial cells from the underlying tissue of the integument, and with it all signs of the newly pigmented area. From the foregoing results, it would appear, therefore, that the factors responsible for the development of pigmentation and spotting during metamorphosis are localized within the integument itself and that the latter is therefore specific in this respect.

MECHANISM OF TAIL-SKIN HISTOLYSIS DURING METAMORPHOSIS

Series D consisted of thirty-five cases of autoplastic skin transplantations which were of two types. The first type involved an interchange of integument from the back with a similar section from the tail (Fig. A, *B*, *T*). The second type consisted of an interchange of a section of skin from the junction of the tail and body with a similar graft from the back (Fig. A, *ST*, *B*). Of this series, twenty-one animals survived metamorphosis, the rest being killed for histological study at various metamorphic stages. Sixteen of these were of the first type and five of the second type of transplantation. In all cases the transplantations were made from 2 to 4 weeks prior to

the onset of metamorphosis, which allowed sufficient time for complete healing of the transplant. The time required for healing was the same as that noted in Series A, B, and C, the grafts regaining their normal appearance in about 1 week, indicating that normal circulation had again been restored to the grafted tissue. The grafts invariably retained their normal pigmentation up to the stage of metamorphosis at which integumentary spotting normally occurs.

The results of Series D emphasized the local specificity of integument as regards pigmentation during metamorphosis, as was shown in Series A, B, and C. Back skin transplanted to regions on the tail developed the normal pigmentary characteristics typical of the region from which it had been taken. Instead of histolyzing and being absorbed in consequence of its contact with atrophying tissue during metamorphosis, as would be expected, it invariably developed the normal pigment pattern characteristic of back skin. The normal greenish coloration and dark pigmented spots appeared with the same intensity as that typical of normal back skin (Plate I, Fig. 5). However, with the resorption of the tail and its integument, the grafts migrated from their original transplanted position to one adjacent to the tip of the urostyle, where they remained indefinitely. Grafts transplanted from the back to the posterior end of the tail underwent the same pigmentary changes during metamorphosis as those transplanted nearer the anterior region. The pigmentary changes of such grafts were found to take place simultaneously with the spotting of the back.

Histological examination of the grafts showed no signs of cellular histolysis, the integument having the appearance of normal back skin in every respect. Plate II, Figure 6, represents a typical section made from such a graft removed at a stage when the tail had been reduced to 1 cm. in length. The *stratum compactum* layer of the host skin, it will be noted, has undergone an almost complete degeneration, the epithelial cells being crowded together somewhat while the pigment layer beneath has become more compact. The graft, however, appears normal in every respect, and a very definite junction line appeared between it and the histolyzing skin of the tail.

The reciprocal graft, however, in which tail skin was transplanted to the back, underwent degeneration, during metamorphosis, and

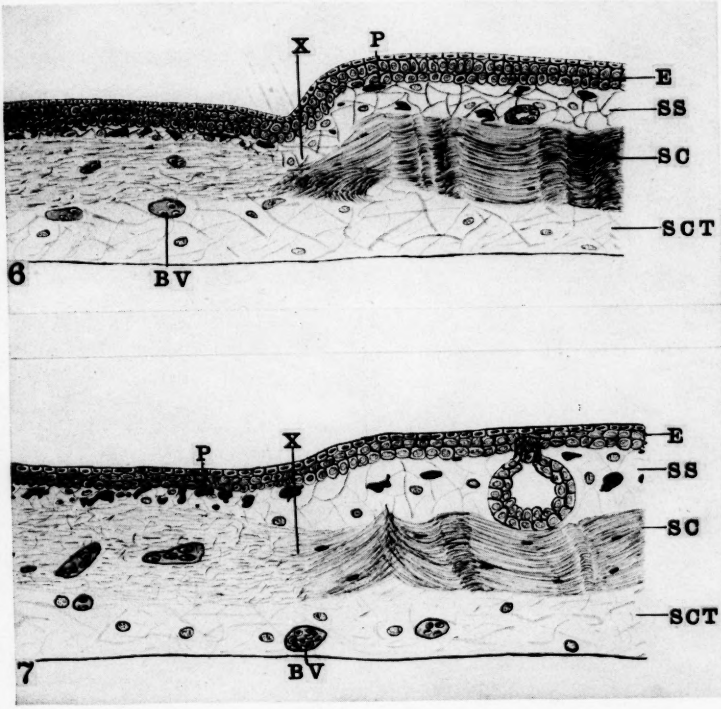
PLATE II

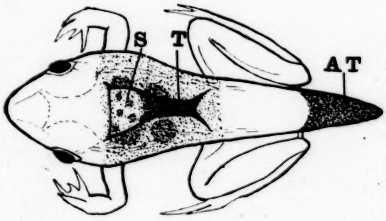
FIG. 6.—Section through back graft transplanted on tail and of adjacent atrophying tail integument. *E*, epidermis; *SS*, *stratum spongiosum; SC*, *stratum compactum; SCT*, subcutaneous connective tissue; *P*, pigment masses; *BV*, blood vessels; *X*, junction of back graft and atrophying tail skin. *Stratum compactum* dissociated, crowding together of epithelial cells and pigment masses, of tail integument. Graft normal in every respect.

FIG. 7.—Section through tail graft transplanted to back. Note *stratum compactum*, pigment masses, and epithelial cells. Showing normal histolysis of tail integument when transplanted to the back. *X*, junction of tail-skin graft and back skin.

FIG. 8.—Graft obtained from junction of tail and side. *S*, side portion; *T*, tail portion; *AT*, atrophying tail. Macroscopic appearance of graft during metamorphosis. Tail portion histolyzing; side portion developing normal pigmentation.

PLATE II





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presented all the external signs of histolysis. When transplants of tail skin were made to the back, the normal process of healing took place, as was found for other types of skin grafts. Such grafts retained their normal pigmentation and apparently acquired a normal blood supply. However, at the stage of metamorphosis when tail skin normally undergoes visible histolysis, the grafts become slightly darker in color corresponding to the darkening of the integument of the atrophying tail. As metamorphosis progressed, the grafts were resorbed, again corresponding exactly with the resorption of the integument of the tail.

Histological examination showed histolysis identical to that typical of histolyzing tail integument during the atrophy of the tail. This will be made clear by reference to Plate II, Figure 7, which depicts normally histolyzing tail skin. In this connection, it may be said, a complete study was not undertaken regarding the histological sequence of events which take place in this type of histolysis, the study being more from a macroscopical point of view. It may be said, however, that the microscopical picture of tail-skin histolysis obtained by the writer compares quite closely with those described and pictured by Helff (1926), for opercular integument.

Table II, Figure 8, represents a second type of tail-skin transplantation, in which a graft obtained from the junction of the tail and side of body, including skin from both regions, was interchanged with a back-skin graft. Similar to the foregoing results, an absorption of the tail integument occurred, during metamorphosis, while that of the side developed into normal side skin. Histological sections of such grafts were similar to those represented in Figures 6 and 7, the tail-skin portion of the graft always undergoing typical histolysis while the side-skin part remained normal in all respects.

DISCUSSION

The results of skin transplantation experiments have served to emphasize certain fundamental principles involved in the mechanisms of integumentary metamorphosis. An important point which has been frequently emphasized is that of specificity of pigmentation. The work of Uhlenhuth (1918) in this regard is of interest. He concluded that two factors are involved in the development of pigmen-

tary spots in *Amblystoma*, one factor which is contained within the skin itself, and a second, which he calls "metamorphosis factor," contained not in the skin but in the circulation. He states regarding the latter, "It may be identical with an agent like the thyroid substance." Weigl (1913), however, believed that in the salamander the metamorphosis of the skin is a process of self-differentiation.

The present work indicates quite strongly that the factors responsible for the development of pigmentation in the frog during metamorphosis are from more than one source. From the results obtained, it seems quite probable that the presence of a specific hereditary factor within the skin itself cannot be denied. In other words, the various areas of integument of the tadpole have latent within them the specific pigment cells or factors to develop, at a certain stage of metamorphosis, the pigment pattern characteristic of the adult. The development of the characteristic pigmentation pattern in integument when transplanted to foreign locations would seem to fully substantiate such a conclusion.

At the same time, however, it is logical to assume the presence of some factor, or factors, within the blood stream which is active or becomes active at a certain stage of metamorphosis. Such a factor, or factors, might well be conceived as being responsible for the rapid development of the latent pigmentation pattern present in the various areas of the integument.

The results of the present work serve to throw light on the possible specificity and mechanisms of tail-skin histolysis as it occurs during the normal atrophy of the tail during metamorphosis. It has been suggested by Barfurth (1887) and Bataillon (1891) that atrophy of the tail is induced primarily by the rapid growth of the pygostyle during metamorphosis, which serves to at least partially occlude the blood supply to the tail. This, according to Morse (1918), would be a possible explanation for the condition of acidosis which occurs in the tissues of the atrophying tail. Morse, furthermore, points out that the acidosis which is present is sufficient to bring about autolytic reactions, which in turn result in the atrophy of the tail.

Although the foregoing explanation might serve to explain the degeneration of certain tissues of the tail, particularly muscular

tissue, it cannot be accepted by the writer when applied to the histolysis of tail integument during metamorphosis. The fact that tail integument transplanted to the back undergoes normal histolysis without the presence of adjoining atrophying tissue requires some other explanation. Moreover, the fact that integument from the back transplanted to the tail never undergoes histolysis during tail atrophy, is a negation of the idea that tail skin undergoes histolysis during metamorphosis entirely because of its association with the atrophying musculature beneath. In this connection the work of Helff (1926) is of interest. The latter author induced histolysis of opercular skin grafts transplanted to the back by insertion of atrophying tail muscle beneath such grafts. Although concerned primarily with histolysis of opercular integument as it occurs during the formation of the opercular fore-limb perforations during metamorphosis, the preceding experiment led Helff to suggest the probability that histolysis of tail integument, as it normally occurs, might be due to the presence of the underlying atrophying musculature of the tail. The results of the present paper indicate clearly, however, that complete histolysis of tail skin can take place when transplanted to a foreign location and without association with other atrophying tissue of the tail.

Therefore the mechanism of tail-skin histolysis as it occurs during metamorphosis would appear to be a twofold one. The integument of the tail must be specific, hereditarily, to undergo a process of histolysis when subjected to certain influences during metamorphosis. Secondarily, the results justify the assumption that this influence must be transported through the blood stream and is liberated or becomes active in the latter at a definite stage of metamorphosis. The exact nature of this influence, although probably hormonic, is quite problematic.

SUMMARY AND CONCLUSIONS

1. Several types of larval skin-transplantation experiments were made in an attempt to determine the local specificity of frog integument when transplanted to foreign regions and allowed to metamorphose. The grafts involved skin from the back, belly, side, and

tail. Observations were made with respect to the specificity of integument for pigmentation and histolysis.

2. Forty-one cases of autoplastic and homoplastic larval skin grafts showed local specificity for pigmentation when allowed to metamorphose. All grafts invariably developed the pigment pattern characteristic of the region from which they had been removed. It is concluded that the integument of the frog is specific for pigmentation, and that the factor responsible for the development of the normal pattern is contained within the skin itself.

3. Similar transplants involving an interchange of tail skin with skin from the back suggested the specificity of tail skin toward histolysis. Tail skin, when transplanted to the back, undergoes histolysis during metamorphosis. Conversely, skin transplanted from the back to the tail undergoes normal development during metamorphosis and develops the pigmentary pattern characteristic of the region from which it was taken. It is concluded that two factors determine the histolysis of tail integument, one of which lies within the skin itself and is specific, and the second, within the blood stream, which becomes functional in inducing histolysis of tail integument at a certain stage of metamorphosis.

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MALE PRODUCTION IN CROWDED AND UNCROWDED CULTURES OF THE ROTIFER HYDATINA SENTA[1]

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IN THE Cladocera, Banta and Brown have found that the mothers which are kept in crowded cultures produce a higher proportion of males from the parthenogenetic eggs than the mothers in uncrowded cultures. They think the increased concentration of excretory products from the animals in the crowded cultures brings about the increased male production. They also find that the addition of carbon dioxide or chloretone or uric acid to the culture water will increase the percentage of males. They conclude that these conditions lower the rate of metabolism of the mothers and in some way stimulate the production of males.

Shull, on the contrary, has found that in the rotifer *Hydatina senta*, old culture water of horse manure, which presumably contains considerable excretory products, reduces the percentage of male-producing daughters. He also finds that the production of male-producing daughters may be reduced by the addition to the culture water of such substances as urea; beef extract; creatin; four ammonium compounds, as the hydroxide, chloride, carbonate, and nitrate; as well as potassium sulphate and iron chloride.

Shull, Whitney, and Riddle have suggested that this increase in the male-producing females in *Hydatina senta* is correlated with an increased rate of metabolism. These tentative suggestions were based largely upon general observations rather than upon extensive specific experiments. Whether this increased rate of metabolism causes the increased production of male-producing daughters or appears as a result of this greater production of male-producing daughters has never been determined.

Acting upon the suggestion of Dr. Banta that some definite crowding experiments be tried with *Hydatina senta*, the author ac-

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cepted the invitation of the Station for Experimental Evolution of the Carnegie Institution and spent several weeks at Cold Spring Harbor during the summer of 1928. The author is deeply grateful to the director and to Dr. Banta for all the necessary facilities and equipment which made possible the completion of the experiments.

Two parallel series of experiments were made in which all the initial factors were identical excepting the food supply. In one series the colorless flagellate *Polytoma* was used as food. In former experiments this protozoan has been used extensively for the production of a high percentage of female-producing daughters when it was raised in horse-manure solutions. In the present experiments it was raised in poultry-bone-meal solution, made by adding a muslin bag of 100 grams of bone meal to 1,000 cc. of rain water, the old bag of meal being removed every 48 hours. Enormous numbers of the *Polytoma* would develop and form an orange-colored scum on the surface of the culture solutions. This was removed, put into tubes, centrifuged, water poured out, new water added, and centrifuged a second time to insure a thorough washing off of all bone-meal solution, which if not removed is detrimental to the rotifers.

The other series of the parallel set were fed upon *Euglena*, which were raised in a split-pea and citric-acid culture solution as suggested by Baker. Four to six yellow, dried split peas were added daily to 600 cc. of rain water to which 8 drops of a normal solution of citric acid had been added. Enormous numbers of *Euglena* were developed in 2–3 weeks. These were washed in the same manner as were the *Polytoma*. In former experiments it has been noted that *Euglena* as a food causes a much higher production of male-producing daughters than the *Polytoma*. These two kinds of foods, one retarding and the other stimulating the production of male-producing daughters, were selected in order to better observe the effects of crowding in the midst of such contrasting conditions.

The experiments were conducted at a temperature of 21°–23° C. Small slender shell vials of $1\frac{1}{4}$ cc. capacity were used in the early experiments; but later, broader 7 cc. shell vials (20×30 mm.) were found more satisfactory. In some of the experiments, 50 females were put into 0.5 cc. of culture water; and in others, 100–200 females were put into 1 cc. of culture water. Food was added every 12 hours.

In the best experiments the females multiplied rapidly, and at the end of 72–96 hours there were 1,000–1,600 living females in this small quantity of water. At the end of this period, in some of the

TABLE I

Polytoma USED AS FOOD. SIXTEEN CROWDED EXPERIMENTS COMPARED WITH 8 UNCROWDED CONTROL EXPERIMENTS, SHOWING IN MOST CASES A LOWER PERCENTAGE OF MALE-PRODUCING FEMALES PRODUCED IN THE CROWDED EXPERIMENTS THAN IN THE UNCROWDED ONES

EXPERIMENT	AMOUNT OF CULTURE WATER (CC.)	NO. OF FEMALES AT BEGINNING OF EXPERIMENT	NO. OF FEMALES AT END OF EXPERIMENT	TIME OF CROWDING (HOURS)	YOUNG FEMALES ISOLATED AFTER HATCHING FROM EGGS WHICH WERE IN THE DISHES AT TERMINATION OF THE EXPERIMENTS			
					No.	♀♀	♂♀	% ♂♀
Control.....	10.0	10	.....	.....	15	12	3	20.
3.....	0.5	100±	.....	48	20	20	0	0.
4.....	0.5	100±	.....	48	20	20	0	0.
Control.....	10.0	10	.....	.....	16	16	0	0.
5.....	0.5	100±	200±	48	17	15	2	11.+
6.....	0.5	100±	100±	72	20	18	2	10.
Control.....	10.0	10	.....	.....	20	6	14	70.
7.....	0.5	150±	.....	48	20	16	4	20.
8.....	0.5	150±	.....	72	18	17	1	5.+
Control.....	10.0	10	.....	.....	17	14	3	17.+
9.....	0.5	150±	.....	48	20	20	0	0.
10.....	0.5	150±	.....	48	20	20	0	0.
Control.....	10	10	.....	.....	16	6	10	62.+
13.....	1	100±	.....	96	19	14	5	26.+
14.....	1	100±	932	96	18	14	4	22.+
Control.....	10	10	.....	.....	20	16	4	20.
15.....	1	150±	952	96	20	19	1	5.
16.....	1	150±	.....	96	18	18	0	0.
Control.....	10	10	.....	.....	38	36	2	5.+
17.....	1	200±	.....	108	15	13	2	13.+
18.....	1	200±	607	108	18	17	1	5.+
Control.....	10	10	.....	.....	14	11	3	21.+
19.....	1	100±	.....	144	16	16	0	0.
20.....	1	100±	819	144	18	13	5	27.+

experiments, the culture water and the rotifers were poured out, leaving the unhatched parthenogenetic eggs adhering to the sides of the vial. Fresh culture water was added, the eggs allowed to hatch, and the young females isolated to determine the nature of their off-

spring, whether female or male. In other experiments the culture water and the rotifers were transferred to a clean vial 24 hours before the termination of the experiments, so as to obtain eggs that were

TABLE II

Euglena USED AS FOOD. SEVENTEEN CROWDED EXPERIMENTS COMPARED WITH 9 UNCROWDED CONTROL EXPERIMENTS, SHOWING IN ALL CASES A LOWER PERCENTAGE OF MALE-PRODUCING FEMALES PRODUCED IN THE CROWDED EXPERIMENTS THAN IN THE UNCROWDED ONES

EXPERIMENT	AMOUNT OF CULTURE WATER (CC.)	NO. OF FEMALES AT BEGINNING OF EXPERIMENT	NO. OF FEMALES AT END OF EXPERIMENT	TIME OF CROWDING (HOURS)	YOUNG FEMALES ISOLATED AFTER HATCHING FROM EGGS WHICH WERE IN THE DISHES AT TERMINATION OF THE EXPERIMENTS			
					No.	Female-producing Females	Male-producing Females	% Male-producing Females
Control.....	10.0	10	.....	.....	7	1	6	85.+
1.....	0.5	100	175±	48	20	13	7	35.
2.....	0.5	50	200±	72	20	17	3	15.
Control.....	10.0	10	.....	.....	20	7	13	65.
5.....	0.5	100	300±	48	20	20	0	0.
6.....	0.5	100	413	72	20	13	7	35.
Control.....	10.0	10	.....	.....	18	4	14	77.+
7.....	0.5	150	.....	48	20	12	8	40.
8.....	0.5	150	.....	72	20	15	5	35.
Control.....	10.0	10	.....	.....	4	3	1	25.
9.....	0.5	100	All dead	48	.....	.....	.....	.....
10.....	0.5	100	All dead	48	14	14	0	0.
Control.....	10.0	10	.....	.....	39	25	14	35.+
11.....	0.5	150	.....	48	20	20	0	0.
12.....	0.5	150	.....	72	19	15	4	21.+
Control.....	10	10	.....	.....	17	7	10	58.+
13.....	1	100±	.....	96	20	19	1	5.
14.....	1	100±	.....	96	20	20	0	0.
Control.....	10	10	.....	.....	15	6	9	60.
15.....	1	150	1,696	96	10	10	0	0.
16.....	1	150	.....	96	15	14	1	6.+
Control.....	10	10	.....	.....	19	12	7	36.+
17.....	1	200±	.....	108	20	19	1	5.
18.....	1	200±	1,173	108	16	15	1	6.+
Control.....	10	10	.....	.....	17	16	1	5.+
19.....	1	100±	.....	144	20	20	0	0.
20.....	1	100±	815	144	20	20	0	0.

laid during the last few hours when the crowding was at its greatest intensity. Experiments 13–18 were made in this manner.

The experiments in Tables I and II, with corresponding numbers, were conducted in parallel sets with identical conditions, with the exception of the foods and their resulting influences. Both tables show that the uncrowded controls produced the greater percentage of male-producing daughters and that the longer the crowded conditions prevailed the lower the percentage of male-producing daughters became. Those fed upon *Euglena* thrived better and multiplied faster than those fed upon *Polytoma*. This was due probably to the production of oxygen by the *Euglena*, better living conditions in the culture water thus being developed.

Table III shows the summarized results of Tables I and II, and here the general results very clearly show that crowding reduces the relative numbers of the male-producing females.

TABLE III

SUMMARY OF TABLES I AND II, SHOWING THE MARKED REDUCTION OF MALE-PRODUCING DAUGHTERS IN THE CROWDED EXPERIMENTS

FOOD	EXPERIMENTS	YOUNG FEMALES ISOLATED AFTER HATCHING FROM EGGS WHICH WERE IN THE DISHES AT TERMINATION OF EXPERIMENTS				
			No.	Female-producing Females	Male-producing Females	% Male-producing Females
Polytoma	8	Uncrowded controls	156	117	39	25.00
	16	Crowded	297	270	27	10.00
Euglena	9	Uncrowded controls	156	81	75	48.07
	17	Crowded	314	276	38	12.10

No definite observations were made to determine whether the rate of metabolism was lowered or raised by crowding. In previous observations by Whitney and Shull, it has been found that an adult female rotifer of this species lays 8–12 eggs every 24 hours for 3–4 days in succession. These eggs hatch and the young females grow and mature and themselves are laying eggs by the end of 36 hours. In the thirteen experiments of Tables I and II, in which the rotifers were counted at the end of the 48–144-hour crowding periods, it can be noted that rate of egg production could not have been more than 2–4 eggs a day instead of 8–12 eggs daily as under normal conditions.

This would indicate a lowered rate of metabolism induced by crowding. Moro has found that there are some indications in the rotifer *Branchionus pala* that suggest that the male-producing daughters may be produced faster than the female-producing daughters.

This decrease in male production when the rate of metabolism is lowered by crowding in *Hydatina senta* is just the reverse of what Banta and Brown have found in the Cladocera. These differing results may indicate either that the lowered rate of metabolism brought about by crowding in Cladocera and rotifers is an incidental phenomenon and has no influence in stimulating or repressing male production or that the stimulating causes of male production differ in the various groups of parthenogenetic animals.

SUMMARY

In crowded cultures of the rotifer *Hydatina senta* in which the rate of metabolism is apparently lowered the production of males is markedly reduced.

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THE EFFECT OF TEMPERATURE ON THE OXYGEN CONSUMPTION OF AN INSECT, MELANOPLUS DIFFERENTIALIS

(Two figures)

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INTRODUCTION

EXPERIMENTAL investigations on the effect of temperature on insects have been concerned principally with development (Krogh, 1915; Bodine, 1925; Bliss, 1926; Ludwig, 1928; and others). Most of the experiments upon the effect of temperature on oxygen consumption in insects give results of exposure either to a series of temperatures or to a constant temperature (Slowtzoff, 1909; Batelli and Stern, 1913; etc.). In many cases the insects have been used in groups rather than singly (Parhon, 1909; Slowtzoff, 1909; Orr, 1925; von Buddenbrock and von Rohr, 1921).

The purpose of the present paper is to give a report of quantitative experiments on the effect of temperature, in a range of 10°–52° C., upon the oxygen consumption of single grasshopper nymphs (*Melanoplus differentialis*).

ACKNOWLEDGMENT

The writer wishes to express appreciation to Dr. J. H. Bodine for the valuable advice and inspiring criticism which made the work possible.

MATERIAL AND METHOD

Male and female nymphs between the second and fourth instar were used. The weights of the insects ranged between 0.093 and 0.338 grams, and the length measurements ranged between 12 and 20 mm.

The instruments for measuring the oxygen consumption were modified Krogh manometers of the type described by Bodine and Orr (1925). The temperature was controlled to $\pm 0.5°$ C. by the use of a water bath in which the water was constantly stirred.

The wire basket containing the insect when it was placed in the manometer bulb was small enough to assure that possible muscular movements of the insect were at a minimum.

Since male and female nymphs were found to give similar values for the rates of oxygen consumption, both sexes were used interchangeably.

During the winter and spring of 1928 thirteen nymphs were exposed to a series of temperatures. An important fact to be noted is that the insects were not used in groups, as has been done by most investigators, but each animal was separately exposed to a series of temperatures. As an example of the type of series used, the following may be named: 10°, 21°, 32°, 41°, 51°, and 21°, 31°, 41°, 51°, and 21°, 26°, 31°, 36°, 41°, 47° C., etc. Each insect was exposed to each temperature at least 10 minutes before readings were taken, and in the majority of experiments the first reading for each temperature was discarded; the purpose of this procedure was to insure that the animal was accustomed to the temperature. The length of exposure to each temperature after the first reading was taken was 1 hour, more or less. The readings were taken at 5-minute intervals; therefore the final value determined was an average of many points taken over the whole interval.

During the summer of 1928, one hundred and five nymphs were used, which were separately exposed to the various temperatures as had been done in the spring and winter. Each animal was subjected to but one temperature instead of to a series of temperatures as had been done previously. The remainder of the procedure was identical with that carried on during the winter.

RESULTS

The average rate of oxygen consumption by the grasshopper nymphs at approximately room temperature, 21° C., was found to be 7.1 cubic millimeters of oxygen per gram per minute (Fig. 1).

When the insects were exposed to a series of gradually increasing temperatures, the rate of oxygen consumption increased with increase in temperature, up to 47° C., as is shown by Table I. The increase in rate is not directly proportional to increase in temperature.

When the insects were exposed to single temperatures, the rate

of oxygen consumption increased with increase in temperature, as shown by Table II; but there is an important difference from the results of exposure to a series of temperatures. Above 15°c. the values for the rate of oxygen consumption at single temperatures

TABLE I

Temperature	Rate of O_2 Consumption per Gram per Minute
15° C.	4.9 cu. mm.
25° C.	8.9 cu. mm.
35° C.	14.8 cu. mm.
47° C.	40.6 cu. mm.
51° C.	36.7 cu. mm.

TABLE II

Temperature	Rate of O_2 Consumption per Gram per Minute
15° C.	4.8 cu. mm.
25° C.	14.1 cu. mm.
35° C.	28.5 cu. mm.
47° C.	41.6 cu. mm.
51° C.	24.6 cu. mm.

were considerably higher than the values for the rate as a result of exposure to a series of temperatures.

All of the average rates of oxygen consumption for each temperature have been plotted on Figure 1, and a curve has been drawn which is a mean of all the average rates. Since this curve rises gradually from the low point of 2.2 cu. mm. at 10° C. to the high point of 41.6 cu. mm. at 47° C, it shows that there is an increase in oxygen consumption with increase in temperature up to a certain limit. Beyond that limit, 47° C., the curve drops abruptly, and indicates that 47° C. is the limit of normal physiological reaction for the insect.

The points plotted show clearly that the rates of oxygen consumption are considerably higher for nymphs exposed to single temperatures than for nymphs exposed to a series of temperatures.

In all cases the grasshoppers lived after exposure to 47° C. but died after exposure to 51° C. These results are consistent with the statements by Arrhenius (1915) that protoplasm suffers at temperatures above 40° C., that it is killed at 55° or 60° C., and that vital processes become hampered at temperatures above 40° C.

DISCUSSION

At present there are three principal theories concerning the effect of temperature on physiological reactions; namely, those of van't Hoff, Arrhenius, and Krogh.

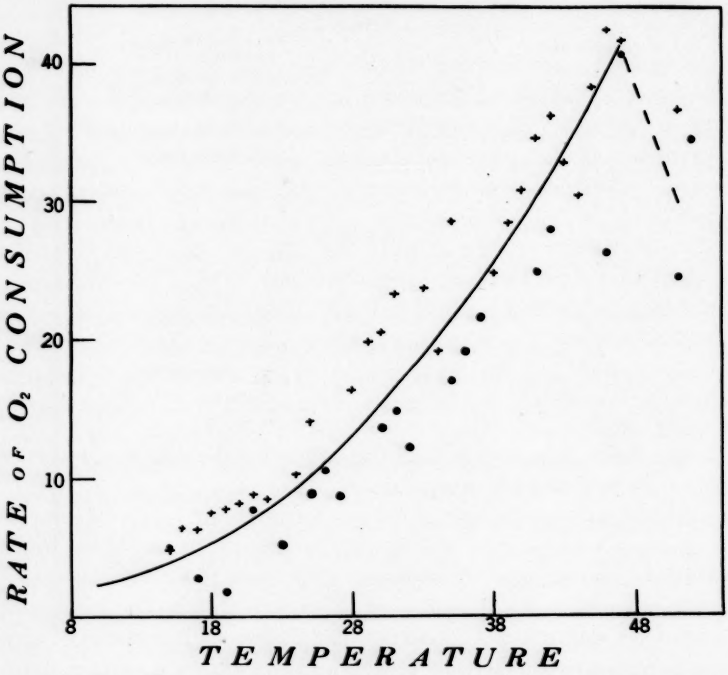

FIG. 1.—Effect of temperature on the rate of oxygen consumption of grasshopper nymphs. X = nymphs exposed to a single temperature. ● = nymphs exposed to a series of temperatures. Points represent averages of observed values.

Van't Hoff's theory (1884) is essentially a theory for the effect of temperature on chemical reactions, but it has been applied to various physiological reactions for the purpose of determining if chemical reactions and physiological reactions are similar. According to van't Hoff, for every 10° increase in temperature the rate of a chemical reaction is increased from two to three times. The hypothesis may be expressed in the following formula:

$$Q_{10}=\left(\frac{K_2}{K_1}\right)^{\frac{10}{t_2-t_1}},$$

in which Q_{10} is the temperature coefficient; K_1 is the constant for the rate of the reaction at one temperature, t_1; and K_2 is the rate of the reaction at a second temperature, t_2. Van't Hoff (1884) found that for many chemical reactions the temperature coefficient, Q_{10}, was constant; and when it decreased with increase in temperature, he suggested the cause to be changes in the viscosity of the solvent when temperature was increased.

Various investigators, among whom are Hertwig (1898) and Loeb (1908), in studying the effect of temperature on growth and development, found temperature coefficient values of more than 2 and concluded that the processes were chemical in nature. Other investigators, Krogh (1914), Chapman (1925), and Ludwig (1927), find that Q_{10} values decrease with increase in temperature and that van't Hoff's formula cannot be applied.

Arrhenius (1915) is of the opinion that the effect of temperature on physiological processes follows the same law as is found for the influence of temperature on ordinary chemical processes, but his conception differs from that of van't Hoff (1884). His theory may be briefly stated as follows: The velocity of a reaction at one absolute temperature equals the velocity at a second absolute temperature multiplied by the logarithm to the base e. Expressing this in a formula, we have

$$\frac{K_2}{K_1}=\frac{\mu\left(\frac{1}{t_1}-\frac{1}{t_2}\right)}{e^2},$$

where K_1 is the velocity of the process at first temperature, t_1; K_2 is the velocity of the process at a second temperature, t_2; μ is the temperature characteristic and is constant for the process in question; and t_1 and t_2 are absolute temperatures.

Arrhenius (1915) determined that for many chemical and biological reactions μ was constant and that in some cases it was the same for chemical and biological processes. Krogh (1914) found that for the effect of temperature on cleavage of the frog's egg, Arrhenius' formula could not be applied. Ludwig (1927) found that it could not be applied to the development of the Japanese beetle. Crozier (1924) extended Arrhenius' work and plotted critical thermal incre-

ments for various physiological processes. He determined the critical thermal increment for biological oxidations to be of two or three types; namely, μ 11,500 and 16,000 or 16,700. The first value is for temperatures above 15° C., and the second is for temperatures below 15° C. There was a break in the straight-line type of curve at 15° C.; this point was called the "critical" point. Orr (1925), in his work on *Drosophila melanogaster* pupa, found the values of μ to be 11,500 above 15° C. and 16,800 below 15° C. His curve was a straight line with a "break" at 15° C.

The third theory is that of Krogh (1915). For growth and development of embryos, he found that neither van't Hoff's nor Arrhenius' theories fit the process in question, and he formulated an algebraic statement, $Vt+10°=Vt+K_{10}$, in which increment in rate is proportional to increment in temperature. Krogh's theory has been given additional quantitative proof by Bodine (1925) for the development of certain Orthoptera eggs and by Ludwig (1927) for the development of the Japanese beetle.

The majority of experiments which prove or disprove the aforesaid theories have been investigations on such phenomena as the growth and development of organisms (Hertwig, 1898; Loeb, 1908; Bliss, 1926; Ludwig, 1928) or heart-beat frequency (Martin, 1904), or respiratory movements of insects (Crozier and Stier, 1924) or the oxygen utilization of insect pupae (Krogh, 1916, with *Tenebrio* pupae). The experiments of von Buddenbrock and von Rohr (1921) upon the walking-stick (*Dixippus morosus*) are quantitative studies of the effect of temperature on the oxygen consumption of groups of adult insects. They found that only Kroghs' formula could be applied, since their result was a straight-line curve for the temperature 10°–30° C.

In an attempt to apply van't Hoff's theory (1884) to the present results, it was found that the values for the temperature coefficient Q_{10} decrease with increase in temperature (Table III).

TABLE III

Temperature	Q_{10} Value
10°–20° C.	2.91
20°–30° C.	2.40
30°–40° C.	1.86
40°–50° C.	1.14

In accordance with Arrhenius' theory, a critical thermal increment curve was plotted (Fig. 2) from the mean values of all the observed rates. It is a curve of the type pointed out by Crozier (1924) and also determined by Orr (1925) for oxygen consumption of *Drosophila* pupae. The curve is a straight-line type with a "break" occurring at 30°; above 47° the curve drops off, indicating the limit of normal physiological reaction. The values for the

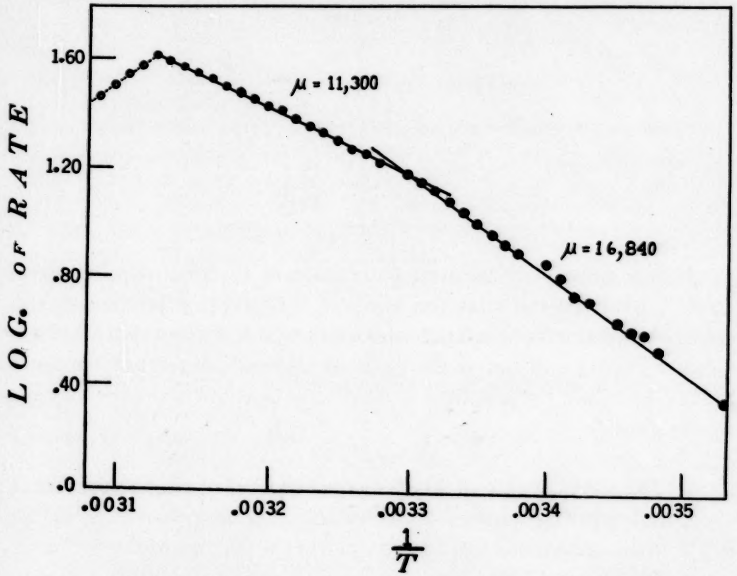

FIG. 2.—Logarithm of rate of oxygen consumption for different temperatures

critical thermal increment are $\mu = 11{,}300$ for temperature 47°–30°, and $\mu = 16{,}840$ for temperatures 30°–10°. It will be remembered that Crozier's values (1924) were $\mu = 11{,}500$ above 15° and $\mu = 16{,}100$ or 16,700 below 15°, and that Orr's values (1925) were $\mu = 11{,}500$ above 15° and 16,800 below 15°. Arrhenius' theory (1915) as extended by Crozier (1924) can be applied to the effect of temperature on the oxygen consumption of the grasshopper nymphs.

As is shown by Table IV, Krogh's formula (1915) does not apply to the experiment in question because the calculated and observed

values vary so widely. The curve of the experimental points is not a straight line as Krogh's theory demands (Fig. 1). Therefore, although Krogh's theory is acceptable for the interpretation of effect of temperature on the rate of growth and development of embryos (Krogh, 1915), and of the Japanese beetle (Ludwig, 1928), it cannot be applied to the effect of temperature on the rate of oxygen consumption of grasshopper nymphs.

TABLE IV

Temperature	$Vt+10°$	
	Calculated Value	Actual Value
10°–20° C.	12.2	6.4
20°–30° C.	16.4	15.4
30°–40° C.	25.4	28.8
40°–50° C.	38.8	33.0

It was previously pointed out that von Buddenbrock and von Rohr (1921) showed that the effect of temperature on the oxygen consumption of adult walking-sticks followed Krogh's theory. When Crozier (1924) calculated the critical thermal increment for their values, he found the μ values to be similar to those demanded by his theory (1924).

CONCLUSION

1. The average rate of oxygen consumption of single nymphs of the grasshopper *Melanoplus differentialis* at a temperature of 21° C. is 7.1 cubic millimeters of oxygen per gram per minute.

2. The effect of increase in temperature is to increase the rate of oxygen consumption. The mean of the averages for all the observed rates is plotted in the form of a curve.

3. Nymphs exposed to but one temperature gives values for the rate of oxygen consumption that, in all cases, are considerably higher than for nymphs exposed to a series of temperatures.

4. The nymphs withstand the temperatures of 47° C., but they die after exposure to 51° C.; 47° C. is approximately the limit for normal physiological reaction for the nymphs.

5. The theories of van't Hoff (1884) and of Krogh (1915) for the effect of temperature on chemical and physiological reactions

cannot be applied; but Arrhenius' theory (1915) as extended by Crozier (1924) fits the process. Two values for μ are found; namely, $\mu = 11{,}300$ at temperature above 30° C. and 16,840 at temperatures below 30° C. These results closely approximate the values found by Crozier (1924) and by Orr (1925).

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